NUCLEOTIDE INHIBITION OF GLYOXALASE II

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The glyoxalase system mediates the conversion of methylglyoxal, a toxic ketoaldehyde, to D-lactic acid. The system is composed of two enzymes, glyoxalase I (Glo-I) and glyoxalase II (Glo-II), and exhibits an absolute requirement for a catalytic quantity of glutathione (GSH). Glo-I catalyzes the isomerization of a hemithioacetal, formed non-enzymatically from methylglyoxal and GSH, to the corresponding α-D-hydroxyacid thioester, s-D-lactoylglutathione (SLG). Glo-II catalyzes the irreversible breakdown of SLG to D-lactate and GSH.

We have observed that ATP or GTP significantly inhibits the Glo-II activity of tissue homogenates from various sources. We have developed a rapid, one step chromatography procedure to purify Glo-II such that the purified enzyme remains "sensitive" to inhibition by ATP or GTP (Glo-II-s). Studies indicate that inhibition of Glo-II-s by nucleotides is restricted to ATP, GTP, ADP, and GDP, with ATP appearing most effective. Kinetics studies have shown that ATP acts as a partial non-competitive inhibitor of Glo-II-s activity, and further suggest that two kinetically distinguishable forms of the enzyme exist.

The sensitivity of pure Glo-II-s to nucleotide inhibition is slowly lost on storage even at -80°C. This loss is accelerated at higher temperatures or in the presence of ATP. Kinetics studies on the resultant "insensitive" enzyme (Glo-II-i) show that a significant reduction of the affinity of the enzyme for the substrate, SLG, occurs and further suggest that only one form of the enzyme is kinetically distinguishable after "de-sensitization".

Tryptophan fluorescence studies of the two enzyme preparations suggest that a subtle conformational change in the enzyme has occurred during de-sensitization.

We have also observed that Glo-II-i is “resensitized” to nucleotide inhibition after incubation in the presence of a reagent that reduces disulfide bonds. The resensitized enzyme exhibits an increased K_M value similar to that of the original Glo-II-s. Kinetics studies show that ATP or GTP again act as partial non-competitive inhibitors of the resensitized enzyme and suggest that only one form of the enzyme is present. The physiological significance of the two enzyme forms is discussed.

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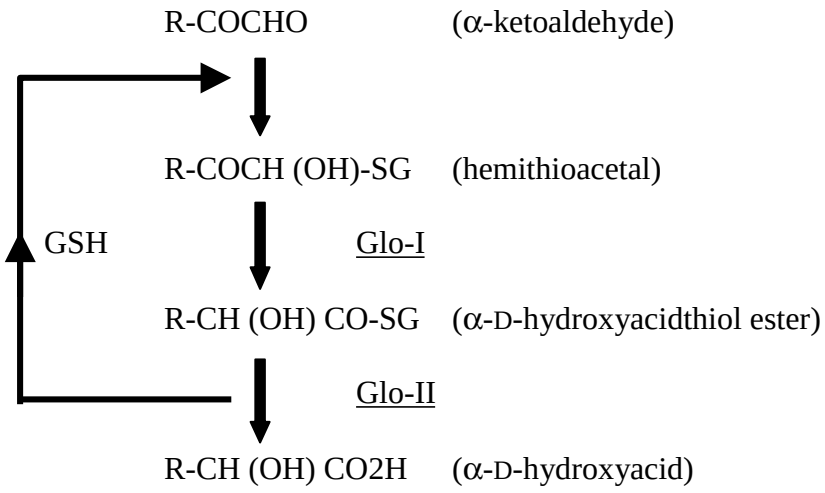
INTRODUCTION

The Glyoxalase system

1.1 Definition

In living systems, the glyoxalase system mediates the detoxification of toxic α-ketoaldehydes by catalyzing their conversion to the corresponding non-toxic carboxylic acids (see Fig. 1). The primary role of glyoxalase activity is considered to be the conversion of methylglyoxal, a toxic α-ketoaldehyde derived from both endogenous and exogenous sources, to lactic acid (1). The system is composed of two enzymes, glyoxalase I (E.C. 4.4.1.5, lactoylglutathione lyase) (Glo-I) and glyoxalase II (E.C. 3.1.2.6, hydroxyacylglutathione hydrolyase) (Glo-II), and exhibits an absolute requirement for a catalytic quantity of reduced glutathione (GSH) (2).



The first step of the glyoxalase system involves the non-enzymatic formation of the physiological substrate of Glo-I, a hemithioacetal from the α-ketoaldehyde and reduced GSH (2).

In the second reaction, Glo-I catalyzes the isomerization of either of the stereoisomers of the hemithioacetal to the corresponding α-D-hydroxyacid thioester (2).

The third and final reaction is an enzymatic hydrolysis in which Glo-II catalyzes the breakdown of the α-D-hydroxyacid thioester to the corresponding α-D-hydroxyacid. Reduced GSH is recycled in the reaction (2).

1.2 History

In 1913, two independent research groups discovered that organisms could readily convert certain α-ketoaldehydes to their corresponding α-hydroxycarboxylic acids (3,4). In particular, the addition of phenylglyoxal or methylglyoxal to the cytosolic extracts from yeast, plant, or animal sources resulted in the production of mandelic or lactic acid, respectively (3-5). Activity was measured as the production of lactic acid either by detecting changes in the optical rotation of the reaction solution (6-7), or by manometric determination of the release of carbon dioxide from a bicarbonate buffered reaction solution following acidification (1,8). This activity, originally thought to be due to the presence of a single enzyme, was named glyoxalase (3, 4).

In 1932, research interests in the field of glyoxalase were motivated by the discovery that GSH was an essential co-factor for the reaction (1). This renewed interest in glyoxalase activity led to the detection of a complex formed from the non-enzymatic reaction of α-ketoaldehydes with reduced GSH (9). This complex, a hemithioacetal, had

been proposed as being the true substrate for glyoxalase activity. An alternative path, in which glyoxalase activity utilizes both GSH and methylglyoxal as substrates in a bi-molecular substrate reaction, has also been proposed (10,11).

In 1936, further research into the mechanism of glyoxalase activity led to the detection of a novel intermediate that was formed by glyoxalase activity subsequent to the addition of methylglyoxal to liver extracts (12). This compound, distinct from that of the hemithioacetal adduct, was proposed as being formed initially from the hemithioacetal and then subsequently converted to the carboxylic acid product by glyoxalase activity. The isolation and identification of this intermediate proved to be an obstacle that impaired further developments in this field for the next fifteen years.

It was not until 1951, when Racker (2) discovered that glyoxalase activity was dependent on the presence of two distinct enzymes, that the pathway from α-ketoaldehydes to their corresponding α-hydroxycarboxylic acids was resolved. Furthermore, he isolated and identified the aforementioned novel intermediate S-D-lactoylglutathione (SLG), the methylglyoxal-derived α-hydroxyacidthioester. This discovery led to the development of a direct spectrophotometric assay for the rate of the formation or of the degradation of SLG that could be utilized to measure the glyoxalase activity of cell extracts.

Racker proposed that the first enzymatic reaction of this pathway involved the conversion of the α-ketoaldehyde/GSH substrate (hemithioacetal) to the corresponding α-D-hydroxyacid-thioester. Support for this proposal was obtained by demonstrating that this reaction, when utilizing methylglyoxal as the α-ketoaldehyde, subsequently produced

the corresponding α-D-hydroxyacid-thioester, SLG. Racker also proposed that the product of the first enzymatic reaction was the substrate for a second enzymatic reaction. Support for this proposal was obtained from demonstrating that the α-D-hydroxyacid-thioester derived from methylglyoxal, SLG, was converted to the corresponding α-hydroxyacid, lactic acid by a second enzymatic reaction. The enzymes involved were named glyoxalase I (Glo-I) and glyoxalase II (Glo-II), the name of each enzyme corresponding to the reaction order in which they appear.

The discoveries of Racker were dominant in stimulating further research interest in the glyoxalase system. Furthermore, his contributions provided many of the basic tools from which further research in this field could be conducted; in fact, the assay methods he developed are still in use today.

1.3 Proposed Functional Roles of the Glyoxalase System

Several possible physiological functions of the glyoxalase system have been proposed over the years. These include a glycolytic bypass, control of the cell cycle, involvement in tubulin dynamics, as well as the detoxification of α-ketoaldehydes.

Originally, the glyoxalase system was proposed as being associated with glycolysis by providing an alternate pathway for the metabolism of triose phosphates to pyruvate via methylglyoxal and lactic acid. This proposal may still carry some merit with respect to prokaryotes, as *E. coli* cells have been shown to convert dihydroxyacetone phosphate to pyruvate via the glyoxalase pathway in an efficient manner (13, 14). In higher organisms, the exclusive production of the L isomer of lactic acid in glycolysis (15), coupled with Racker's discovery that glyoxalase activity produced only the D

isomer of lactate (16) suggested no direct interaction between the two systems. Furthermore, the demonstration that glyoxalase activity is not required for glycolysis to occur shed even more doubt on this proposal (1).

In the 1960's, a research group headed by Szent-Gyorgyi proposed a possible role for the involvement of the glyoxalase system in the regulation of the cell cycle (17, 18). They proposed that the concentration of a universal inhibitory growth factor, a ketoaldehyde derivative they named "retine", regulated the growth of a cell. This factor was reported to be undetectable in rapidly proliferating cancer cells. As ketoaldehydes were the known substrates for the glyoxalase system, it was proposed that glyoxalase might regulate the physiological concentration of such regulatory ketoaldehydes. Decades of research subsequent to this proposal have provided mounting evidence to the contrary but little support for the validity of this theory. Nevertheless, this proposal did stimulate further research and consequently a deeper understanding of the glyoxalase system.

There has been mounting evidence to implicate the involvement of the glyoxalase system in the dynamics of tubulin polymerization, although this evidence remains somewhat ambiguous. In cell free conditions, methylglyoxal has been shown to inhibit the formation of microtubules (19), although only at concentrations far above the reported physiological levels of the metabolite (20). The effect of SLG on the *in vitro* assembly of microtubules is indeterminate, with both the potentiation and the inhibition of microtubule assembly being observed (21). The exogenous addition of SLG to cell cultures has been observed to cause an inhibitory effect upon microtubule-dependent events such as the functional activation of neutrophils (22) and the secretion of histamine

from basophiles (23). Even so, these results should be viewed with caution, as SLG has little membrane permeability and it is believed to be produced and metabolized only in the cytosol of the cell (24). As a number of studies implicated the involvement of the SLG in microtubule dynamics, further research was prompted. Norton *et al* (25) synthesized a number of highly specific Glo-II inhibitors that acted as very slow substrates for the enzyme. Of those inhibitors synthesized, the N,S-bisflourenylmethoxycarbonyl GSH (DiFMOC-G), exhibited the greatest affinity for the enzyme. To increase the membrane permeability of this inhibitor, esters of DiFMOC-G were synthesized. It was anticipated that such inhibitors would specifically increase intracellular concentrations of SLG for a period until their degradation. Indeed, these inhibitors exhibited profound inhibitory effects on early tubulin dynamics (two day cultures) but demonstrated no visible effect after longer incubation periods (15 day cultures).

The possibility of an association of Glo-II and microtubules was also investigated by Norton *et al* (25). Significant thioesterase activity was detected in tubulin preparations even after three association / dissociation cycles of purification. In addition, the thioesterase activity that co-pelleted with the tubulin preparations appeared to undergo changes in substrate specificity. Furthermore, immunochemical techniques have demonstrated a direct physical association of Glo-II with tubulin (25). Presently, the role of the glyoxalase system with respect to tubulin dynamics, if any, remains uncertain.

The major role of the glyoxalase system is considered to be that of methylglyoxal detoxification (26, 27). Methylglyoxal is considered a toxic substance that is cytostatic at low concentrations and cytotoxic at higher concentration (28). This compound is

produced endogenously in a number of different ways, primarily as an unavoidable by-product of triose phosphate metabolism (29-32). It can also be found in the diet of many higher animals (33, 34). Furthermore, a number of strains of bacteria, including enterobacteria, form methylglyoxal enzymatically through the action of methylglyoxal synthase (35). Although other systems exist that are capable of removing methylglyoxal from living systems, the majority of this toxic metabolite is thought to be processed through the glyoxalase system.

1.4 Distribution of the Glyoxalase System

Glyoxalase activity was proposed to be ubiquitous as early as 1945: it has been found at varying levels in all forms of life studied to date including mammals, plants, yeast, and a number of strains of bacteria (3, 4, 5, 35).

The enzymes of the glyoxalase system do not appear to be restricted to any particular tissue in higher organisms, although the specific activity of each enzyme does vary from tissue to tissue. In humans, the highest activity of Glo-I is found in the lung, kidney, pancreas, and brain and is least evident in the liver and adipose tissues. (36). Fetal tissue is reported to exhibit a three-fold increase in Glo-I activity over corresponding non-fetal tissues (36). The activity of Glo-II in humans is greatest in the brain, liver, and muscle, with significantly less activity being present in the spleen and pancreas (37). A number of cancerous tissues are reported to exhibit a major decrease in Glo-II activity when compared to the corresponding non-cancerous tissue (38).

The greater portion of glyoxalase activity is generally found in the cytosolic fraction of cells, although some glyoxalase activity has been reported to exist in cellular

organelles. In particular, Glo-I activity has been detected in permeabilized endoplasmic reticulum (36) while up to 15% of the total Glo-II activity of a cell may be found in mitochondria (39).

1.5 Methylglyoxal

1.5.1 Sources of Methylglyoxal

The physiological substrate for the glyoxalase system is methylglyoxal. In higher organisms, the primary endogenous source of methylglyoxal occurs from the spontaneous elimination of phosphate from triose phosphates, primarily dihydroxyacetone phosphate and to a lesser extent glyceraldehyde-3-phosphate (29). Enzymatic sources of endogenous methylglyoxal include leakage of the bound phospho-ene-diolate from the active site of triose phosphate isomerase (30) and, to a lesser extent, leakage from the active site of the aldolase reaction with glyceraldehyde-3-phosphate (40). Another, although minor endogenous source of methylglyoxal is the action of amine oxidase on aminoacetone, an intermediate in threonine catabolism (41, 42). Still another endogenous source occurs from the conversion of hydroxyacetone, an intermediate in the metabolism of acetone and isopropanol, to methylglyoxal by the action of acetol dehydrogenase (43-45).

Exogenous sources of methylglyoxal in many higher organisms, including humans, are diet and bacteria. Methylglyoxal is an unavoidable consequence of a daily human diet since common beverages and foods such as brewed coffee, cola, juices, toast and tobacco are known to contain significant quantities of methylglyoxal (33, 34). Bacteria provide another source as a number of strains have been shown to produce

methylglyoxal (35), primarily through the action of an enzyme unique to prokaryotes, methylglyoxal synthase. As previously mentioned, enterobacteria have been found to produce methylglyoxal in the same manner (26). This enzyme is inhibited by inorganic phosphate (Pi). Thus it is activated only when the levels of inorganic phosphate are low, and its action frees inorganic phosphate from dihydroxyacetonephosphate to form methylglyoxal (46).

1.5.2 Metabolism of Methylglyoxal

Methylglyoxal can be metabolized by a number of systems other than the glyoxalase system. Other enzymes known to metabolize methylglyoxal include aldose reductase, methylglyoxal dehydrogenase, and methylglyoxal reductase.

Aldose reductase (E.C. 1.1.1.21) catalyzes the formation of hydroxyacetone from methylglyoxal using NADPH as a co-factor (47). Although glyceraldehyde is believed to be the natural substrate for the enzyme, this study indicated that methylglyoxal can also serves as an efficient substrate (47). The enzyme has been shown to exhibit comparable K_M values for these substrates (8 μM for methylglyoxal, 16 μM for D-glyceraldehyde and 42 μM for L-glyceraldehyde), while the k_{cat} / K_M for methylglyoxal (10^7) is one order of magnitude greater than that of glyceraldehyde.

Methylglyoxal dehydrogenase (E.C. 1.2.1.23) catalyzes the oxidation of methylglyoxal directly to pyruvate. It has been isolated from microorganisms, which use NAD^+ as a cofactor (48), and from human liver, which requires either NAD^+ or $NADP^+$ as a cofactor (49). The K_M values exhibited by the enzyme for methylglyoxal are quite high; 4.5 mM and 0.4 mM for the enzymes that require NAD^+ and $NADP^+$, respectively.

Methylglyoxal reductase catalyzes the formation of L-lactaldehyde from methylglyoxal using either NADH (mammalian) (50) or NADPH (yeast) (51) as a co-factor. This enzyme has been purified from goat liver but its involvement in the physiological metabolism of methylglyoxal is unknown.

1.5.3 Methylglyoxal – Concentration and Reactivity

The concentration of methylglyoxal in tissue extracts is assayed by determining the amount of chromophoric adduct formed between the ketoaldehyde and o-phenylene diamine. This procedure requires the separation of the adduct from other interfering chromophores by high-pressure liquid chromatography (52, 53). The concentration of methylglyoxal in tissue samples generally remains in the low micromolar range (<3 μM) but is significantly increased in many tissues from diabetic subjects (20, 54).

Methylglyoxal is considered a highly reactive, toxic metabolite although the method by which this metabolite exhibits its toxic effect is still uncertain. The ketoaldehyde has been shown to react with both nucleosides and nucleotides, preferentially those with guanine residues (55, 60). Furthermore, methylglyoxal has been reported to react with the amino acid residues of proteins, in particular arginyl residues (61-68). Finally, there is evidence that methylglyoxal inhibits the synthesis of cellular proteins (28, 58, 69-71).

As stated previously, methylglyoxal has been shown to bind to both nucleosides and nucleotides, preferentially those that possess the guanine moiety (55, 56). In experiments conducted with this ketoaldehyde to determine the extent of adduct formation with DNA or RNA, no significant binding to DNA was detected until the DNA

was denatured into single strands by heat denaturation. The effect of methylglyoxal on translation was at best weak when using either natural or chemically uncapped mRNA (57, 58). Methylglyoxal has been demonstrated to induce protein – DNA crosslinking in Chinese hamster ovary cells; repair of these moieties occurs within 24 hr. after the removal of the ketoaldehyde from the cell culture medium (59). It was also reported to induce sister chromatid exchange in the same cell line (60). Although the effect of methylglyoxal on nucleotide polymers was significant under these conditions, the concentration of the toxic ketoaldehyde to produce such an effect was far above that of physiological concentrations. Therefore, the proposed role of methylglyoxal as a mutagen or carcinogen in living organisms is probably not realized under conditions where a normally functioning glyoxalase system is present.

Methylglyoxal toxicity appears to be related to its reactivity with proteins (61, 62, 63). It has been shown that at physiological levels, methylglyoxal (1 μM) rapidly forms reversible adducts with arginyl residues of proteins (61). The position of equilibrium suggests that up to 90% of the free α-ketoaldehyde exist in this form (64). The physiological relevance of these adducts has been demonstrated by their prevalence in some disease states. Methylglyoxal concentrations are known to increase significantly in certain disease states such as diabetics, particularly in the lens (20, 54). It has also been demonstrated to bind in a rapid and efficient manner with gamma II-crystallins of both human and animal lens, implicating these adducts in the process of cataract formation, a common diabetic complication (65). Recently, the methylglyoxal-arginyl adducts found in proteins have been demonstrated to act as advanced glycation endproducts (66). The arginine-methylglyoxal adducts proved a potent signal for both the synthesis and

secretion of macrophage-colony stimulation factor(67) as well as the degradation of proteins by monocytic cells (68).

Methylglyoxal toxicity appears to be related to its effect on cell growth as well. The addition of exogenous methylglyoxal to cell cultures is known to inhibit growth, a consequence that can be reversed by the removal of the ketoaldehyde (69). This effect was found to be directly associated with the inhibition of protein synthesis (28), primarily during the S and G_2 phases of the cell cycle (70). Further testing suggests that the effect was not due to any direct interaction with DNA (70) or mRNA (58). Anecdotal evidence have led to the proposal that the inhibition of protein synthesis may be due to the inactivation of either tRNA or ribosomal proteins due to formation of arginyl / methylglyoxal adducts (71).

Much evidence suggests that methylglyoxal can cause damaging effects upon living organisms; however, the efficiency of the glyoxalase system is thought to maintain toxic α-ketoaldehydes at levels below physiological relevance. It is therefore probable that only when an inhibited or impaired glyoxalase system allows chronic levels of methylglyoxal to be sustained that damage may follow.

1.6 S-D-lactoylglutathione

The product of GLO-1 activity with the methylglyoxal derived hemithioacetal produces a high-energy intermediate, SLG. Due to its poor membrane permeability, this intermediate is thought to be produced and metabolized only in the cytosol of the cell (24). Physiological levels of SLG are generally found to be in the micromolar range with

the concentrations of the intermediate being high in liver (>50 μM) and lower in the kidney, lens, and blood (20, 54).

Interest in the role of SLG in biological systems was initially stimulated by the discovery that this intermediate potentiates the polymerization of microtubules *in vitro* (21). As stated earlier (see Section 1.2), although there is evidence that some type of interaction between the glyoxalase system and tubulin does in fact occur, the nature of such an interaction remains unknown.

Further interest in SLG has been motivated by studies that determined the change in SLG concentration during the functional activation of blood cells. Resting neutrophils, upon activation by opsinized-zymosan (phagocytic activation) (72) or 12-O-tetradecanoylphorbol 13 acetate (TPA) (73), undergo 100% increases in SLG levels, independent of aerobic or anaerobic conditions. Furthermore, the addition of exogenous SLG has been shown to potentiate the rate of stimulus-induced secretion of histamine from human leukocytes (23).

1.7 D-lactate

Only about 5% of the total lactate concentration of mammals is in the form of D-lactate (74). A significant source of D-lactate in higher organisms is that produced by the enterobacteria of the gut (75). Another source is that of a normal human diet (76, 77). Although these sources contribute to the total D-lactate content of an organism, the major source is considered to be that produced by the glyoxalase system (78).

In humans, D-lactate is efficiently metabolized to pyruvate in mitochondria by an FAD linked enzyme, 2-hydroxyacid dehydrogenase (E.C. 1.1.99.6) (79). Transport

through cell membranes is accomplished primarily via a specific lactate transporter, although an inorganic anion exchange transport system and a non-ionic diffusion mechanism are known to contribute (80). D-lactate is also known to be efficiently excreted in the urine, sweat, and stool (81 - 83).

The concentration of D-lactate in mammals is highest in the liver and kidney, and lowest in the lens and blood (20, 54, 82). The concentration of this metabolite undergoes 2-3 fold increases in the blood after exercise or meals (77, 81). In diabetic patients, 2-3 fold increases in D-lactate concentrations occur in blood plasma, while in streptozocin-induced diabetic rats, the concentration of D-lactate was found to undergo significant increases in the blood and lens (20, 54, 82).

1.8 Glyoxalase I

1.8.1 Glyoxalase I - Assay of Activity

Glo-I activity has been measured by a number of different methods. One method employs a coupled assay in which Glo-II has been added in excess; consequently the formation of lactic acid becomes dependent on the conversion of methylglyoxal to SLG. The formation of lactic acid can be followed by the manometric determination of the release of CO_2 from bicarbonate buffer after acidification (6,7). A second method involves following the rate at which methylglyoxal is consumed in the reaction by titration of the remaining α-ketoaldehyde with semicarbazide (84).

The most widely used assay procedure to monitor Glo-I activity is the direct spectrophotometric measurement of the formation of SLG at 240 nm

(ε = 3300 M^{-1} cm^{-1}) using methylglyoxal as the substrate (85, 86, 87). This procedure requires the generation of the hemithioacetal by pre-incubation of the ketoaldehyde with GSH (2 mM each), at pH 6.6, resulting in a hemithioacetal concentration of 0.63 mM (Keq=333 M^{-1}) before the addition of enzyme to the reaction mixture (87). The major drawback to this procedure stems from the interference of other compounds in the reaction mixture that add to the total absorbance (ε = 440 M^{-1} cm^{-1} for the hemithioacetal, ε = 115 M^{-1} cm^{-1} for GSH) (87).

1.8.2 Glyoxalase I - Distribution

Glo-I activity has been detected in all tissues of all organisms tested to date and is therefore considered ubiquitous. Activity has been detected in bacteria (88, 89), parasitic protozoa (90, 91), fungi (92), tumors (37), and yeast (93, 94). The enzyme has been extensively purified initially from yeast (2), but has also been purified from the erythrocytes of both rat (95) and pig (26), and from the liver of sheep (96), rabbit (97), and mouse (98). Glo-I activity has also been detected and studied in a number of plants as well (99-101).

Table I summarizes the distribution of Glo-I in humans by both the measurement of specific activity and by radioimmunoassay. In humans, the tissue distribution of Glo-I when determined by radioimmunoassay is highest in the spleen, liver, and adrenal gland and lowest in adipose tissue (36). It is interesting to note that the distribution of the enzyme with respect to specific activity does not correlate with the radioimmunoassay results. Glo-I, when assayed with respect to the specific activity of the enzyme, exhibits

the lowest activities in adipose and liver tissue, while the highest activities are found in the lung, pancreas, kidney, and brain (36). The enzyme exhibits three-fold increases in specific activity in fetal tissue when compared to corresponding adult tissue (36).

Glo-I is thought to be a widespread, ubiquitous enzyme whose activity may be dependent on the proliferative or active state of the tissue. It has been suggested that the activity of the enzyme in plants is regulated by a calmodulin-dependent calcium cascade (99). Inhibition of rapidly proliferating callus growth by calmodulin inhibitors was correlated with a significant decrease in Glo-I activity, an effect that was overcome by addition of endogenous calmodulin or by addition of inositol. Soybean cell cultures that were auxin dependent for proliferation were also observed to undergo increases in Glo-I activity after the addition of auxin to the suspension (101). In mammals, certain disease states have been demonstrated to cause increases in Glo-I activity.

In diabetic patients, activated mononuclear and polymorphonuclear cells from patients, who exhibit diabetic complications, have significant elevation in Glo-I activity (102). Rapidly proliferating cells from colon tumors also show increases in Glo-I activity (103). The activity of Glo-I was also demonstrated to increase in regenerating mouse liver as well (104). Conversely, tissue samples from aged rats, tissues believed to exhibit reduced proliferative capabilities, had a significant decrease in Glo-I activity (105). Further research is warranted to determine the mode by which these changes in activity occur.

1.8.3 Glyoxalase I - Molecular Characteristics

The molecular properties of Glo-I from higher mammals are quite different from those of lower organisms such as yeast and bacteria (see Table II). In all species, Glo-I is a metallo-enzyme that contains one zinc ion per subunit that is required for catalytic activity and / or structural integrity.

The mammalian enzyme exists as a dimer of two similar subunits. In humans, these subunits are expressed at a diallelic gene locus GLO that encodes three possible allozymes, GLO-1 $^{1-1}$, GLO-1 $^{1-2}$, and GLO-1 $^{2-2}$ (106). These allozymes are known to be kinetically indistinguishable (107, 108) but can be separated by ion-exchange chromatography (107) or native gel electrophoresis (109). The estimated molecular weight of the dimer is 46 kDa, as determined by the Stokes radius and sedimentation coefficient (93). The molecular weight of the single subunit has been estimated at 23 kDa by SDS-PAGE (110). In contrast, the enzyme from yeast or bacterial sources exists only in the monomer state with a molecular weight ranging from 31 - 35 kDa (93, 111, 112).

Other differences between the mammalian enzyme and the yeast and / or bacterial enzyme exist as well. One such difference is that of the isoelectric point of the enzymes. The isoelectric point of the yeast enzyme is at neutral pH (113), while the mammalian and bacterial enzymes are acidic enzymes with isoelectric points of 4.8 and 4.0 respectively (114). Another difference in these enzymes is their different affinities for the accepted physiological substrate, methylglyoxal. The K_M values of the mammalian and yeast enzymes, using methylglyoxal as a substrate, are 0.12 mM and 0.53 mM

respectively (107), and are significantly lower than the K_M value of 3.5 mM for the bacterial enzyme using the same substrate (89).

Still another difference in these enzymes lies in the ability of the apoenzymes to be reactivated after removal of the prosthetic zinc ion. The enzyme from all sources contains one Zn^{2+} ion per subunit (115), but, after removal of the ion by chelation, only the mammalian apoenzyme can be reactivated by the addition of zinc. Furthermore, the mammalian enzyme is responsive to metal ion exchange. The apoenzyme from mammalian sources can be reactivated to nearly 100% of the original activity by the addition of magnesium (11, 96, 115-120). Other ions, such as Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, V^{2+}, and Ga^{2+}, can also reactivate the enzyme to a lesser extent. Cd^{2+}, Ba^{2+}, Fe^{2+}, and Hg^{2+} were found to be ineffective (11, 96, 115-120).

Although the enzyme from mammals is considered immunologically distinct from that of yeast and bacteria (36), recent reports show that the amino acid sequence of Glo-I exhibits a large degree of homology throughout nature (121). The primary structure of the yeast enzyme appears to consist of two similar segments with each segment being homologous to the mammalian monomer. This is believed to have occurred due to gene duplication, resulting in a fused dimer. The bacterial enzyme exhibits up to 50% sequence homology with the human enzyme as well (122). Therefore, although the enzymes do exhibit a number of differences, it is likely that all have been derived from a common ancestor.

1.8.4 Glyoxalase I – Substrate Specificity, Kinetics, and Catalytic Mechanism

With respect to substrate specificity, Glo-I accepts a broad range of α-ketoaldehydes as substrates (2, 87, 95, 123-129). The enzyme readily accepts both aromatic and aliphatic compounds of this class regardless of the source of the enzyme, microbial or mammalian. The broad specificity of the enzyme for α-ketoaldehyde substrates is indicative of the role of the glyoxalase system in detoxification. Other detoxification systems such as the cytochrome P-450 system (130) and GSH-S-transferase isozymes (131) exhibit such broad specificities as well.

In contrast to the ability of Glo-I to accept a variety of α-ketoaldehyde substrates, the coenzyme specificity of Glo-I is essentially restricted to the GSH moiety (1). Certain GSH analogs such as homoglutathione (132), isoglutathione, asparthione (133), and others (134, 135) can replace GSH as an active cofactor, although the efficiency of these compounds as cofactors is greatly reduced in comparison to GSH itself. Generally, any hemithioacetal derived from an analog of GSH, which serves as a cofactor for Glo-I activity, exhibits a greatly reduced affinity for the enzyme as reflected in the K_M values for such substrates. It should be noted that although any change in the GSH structure does lower the affinity of Glo-I for the respective substrate, no significant change in the maximum velocity of the enzyme is observed. Therefore the role of GSH with respect to Glo-I activity is thought to be related to enzyme recognition.

The hemithioacetal complex formed from methylglyoxal and GSH is generally assumed as being the substrate for Glo-I, although a two-substrate reaction has never been conclusively ruled out. The assumption for a one-substrate reaction has been based upon “burst” kinetic studies carried out at high substrate concentrations, in which the

addition of increasing levels of Glo-I to assay mixtures did not increase the rate of SLG formation over and above the rate of hemithioacetal formation (126, 136). Although these results support a one-substrate reaction mechanism, studies carried out at low substrate concentrations ($<< K_M$) resulted in non-linear curvature of the Dixon plots indicating that a combination of both one-substrate and two-substrate reactions may be possible (137, 138).

Glo-I follows Michaelis – Menten kinetics over a substrate range up to 0.67 mM in the presence of a constant amount of reduced GSH (139). The values of the Michaelis constant, K_M, are 0.13 mM and 0.53 mM for the mammalian and yeast enzymes respectively utilizing the methylglyoxal-derived hemithioacetal as a substrate (107). The turnover numbers, k_{cat}, range from 59,000-70,000 min^{-1} for the mammalian enzyme and 109,000 min^{-1} for the enzyme from yeast. The catalytic efficiency of the enzymes, k_{cat} / K_M, approaches values of 10^7, a value near the theoretical limits of diffusion. GSH itself acts as a competitive inhibitor of Glo-I activity, although the K_I values, estimated to be as high as 7.9 mM (140) to 18 mM (110), are likely too high to be physiologically relevant. The reaction has been demonstrated to be reversible, although the predicted equilibrium for the reaction lies far to the right in favor of isomerization of the hemithioacetal to SLG (139).

Chemical modification studies have identified a number of possible active site residues. In modification studies, incubation of the enzyme with sulfhydryl group reagents causes the complete inactivation of the enzyme from yeast (141) but have no effect on the activity of the enzyme from mammalian sources (142, 143). Studies

indicate that the enzyme has two free sulfhydryl groups per monomer, four in total for the mammalian dimer and two for the yeast enzyme (93).

Modification of two arginine residues by specific reagents also causes complete loss of catalytic activity (144). The addition of substrate provides effective protection against the loss of activity by preventing the modification of one of the affected arginine residues (144). Results have been interpreted as indicating the presence of an active site arginine essential for catalytic activity.

Modification of Glo-I by tryptophan specific reagents also causes complete inactivation of the enzyme activity (145, 146). One study indicated that a total of three tryptophan residues are modified, the addition of substrate protects the enzyme from modification, and the loss of activity result from the modification of only one of the tryptophan residues (145). Results were interpreted as indicating the presence of an active site tryptophan essential for catalytic activity.

The nitration of tyrosine residues also causes complete inactivation of the enzyme (146). The addition of substrate provides effective protection against the loss of activity (146). It is not yet clear whether the residue modified is involved with the binding of substrate or the coordination of the zinc ion (117).

Other studies have shown that the metal ion is located near the active site (116, 120). Although the presence of a metal ion is required for catalysis, the ability of the mammalian enzyme to accept a number of metal ions while remaining enzymatically active indicates that the role of the metal ion may be to provide structural integrity to the protein.

The reaction catalyzed by Glo-I involves the conversion of the hemithioacetal substrate to the corresponding α-D-hydroxyacid-thioester product via an isomerization reaction. Isotopic experiments have indicated that an exchange of protons from water is unlikely (147, 148); therefore, the isomerization likely involves a shielded proton transfer from the C1 to the C2 of the bound substrate (149), a mechanism which would result in an ene-diol intermediate. Experiments involving isotopic substrates are in agreement with this mechanism (111). Further studies utilizing flavinoid derivatives, compounds known to trap ene-diol intermediates, also support this mechanism (150, 151).

The enzyme has been demonstrated as being able to accept both epimeric forms of hemithioacetal substrates, an advantage that is thought to increase the efficiency of the Glo-I in its role as a detoxification enzyme (149). Although the enzyme is not stereoselective with respect to substrate, Glo-I is known to form only the D isomer of SLG from the methylglyoxal-derived substrate in a stereospecific manner.

1.8.5 Glyoxalase I - Inhibitors

Inhibitors of Glo-I have been investigated for a number of reasons, one being to study the mechanism by which the reaction occurs. Mechanism based inhibitors, such as those that mimic the proposed ene-diol intermediate, have been studied extensively. Such compounds have been demonstrated to be potent Glo-I inhibitors (152, 153). Substituted coumarin derivatives such as isoesculetin and 4-methylcoumarin are examples of ene-diol mimics that potently inhibit Glo-I activity. Flavone derivatives that are also known to trap ene-diol intermediates, such as quercetin, myricetin, and taxifolin, also are inhibitory.

A number of substrate analog inhibitors of Glo-I have been developed as specific agents against the growth of cancerous tissue (154). Glo-II activity has been reported as being greatly decreased in many cancerous tissues when compared to corresponding non-cancerous tissue (37). Theoretically, specific Glo-I inhibitors, which act as slow substrates for Glo-II activity, would be removed efficiently from normal cells by Glo-II. In contrast, cancerous tissues, which possess significantly reduced Glo-II activity, would be unable to hydrolyze the Glo-I inhibitor efficiently, resulting in the specific increase of cytotoxic methylglyoxal in cancerous tissues.

Substrate analog inhibitors of Glo-II include S- and N- terminal substituted derivatives of GSH (154). S- Alkylglutathione derivatives exhibit an increase in inhibitory capability corresponding to an increase in the chain length of the alkyl moiety (154), suggesting the existence of a hydrophobic pocket in the active site of the enzyme which facilitates binding (154, 155). Studies indicate that the N-terminal substituent does not contribute to the specificity of the substrate binding in the mammalian enzyme (155).

1.8.6 Glyoxalase I – Genetics and Cloning

As stated earlier, human Glo-I is expressed at a diallelic gene locus, GLO, which encodes three possible allozymes, GLO-1 $^{1-1}$, GLO-1 $^{1-2}$, and GLO-1 $^{2-2}$ (106, 157). Again, these allozymes are known to be kinetically indistinguishable but can be separated by ion-exchange chromatography (107) or native gel electrophoresis (109). Inheritance of these alleles follows an autosomal diallelic codominant pattern. The two alleles appear in the genomes of both rat (158) and mouse (159) as well. The GLO-1 ^{2} allele is thought to have been the original gene in the genome, while the GLO-1 ^{1} allele is thought to have

occurred due to gene duplication followed by mutation (160). The gene for Glo-I is found on chromosome 6 and is tightly linked to the Human Leukocyte System A (HLA) gene complex (161) in humans, the H-2 gene complex in mice (159), and the Major Histocompatability Complex in rats (158). The expression of the alleles in humans seems to follow specific geographic regularity in that the frequency of the GLO-1^1 allele is extremely low in Asian populations, exists at medium frequencies in African populations, and appears at the highest frequencies in Caucasian populations (162).

The cDNA of Glo-I from a human colon library has been isolated and sequenced and has been demonstrated to express a 180 amino acid protein corresponding to the monomer subunit of the enzyme. The gene has also been successfully cloned into a pMT2 vector and transfected into COS-1 cells (163). The cDNA from *Saccharomyces cerevisiae* has also been cloned into a Yep13 plasmid and successfully transformed into haploid *Saccharomyces cerevisiae* cells (164). The cDNA from *Pseudomonas putida* has also been isolated and cloned into a pBR322 vector plasmid that was utilized to transform *E. coli* cells (165). The amino acid sequences as well as the sequence of the corresponding genes all exhibit strong regions of homology and are therefore likely derived from a common ancestor.

1.9 Glyoxalase II

Glo-II is one of at least three known thioesterases which are specific for the GSH moiety. Others include S-formylglutathione hydrolase (166) and S-succinylglutathione hydrolase (167). Both of these enzymes have been detected in the liver (166) and red blood cells (167) of humans. Studies have indicated that others may occur in nature but the prevalence of these enzymes is as yet unknown. Still, the most prevalent GSH specific thioesterase known is Glo-II and it is therefore considered as the model for thioesterase activity in living organisms.

1.9.1 Glyoxalase II – Assay of Activity

Glo-II activity may be assayed by following the decrease in absorbance at 240 nm (ε = 3.37 mM^{-1} cm^{-1}) due to the hydrolysis of the substrate, SLG (168). A second widely used method follows the production of GSH from the hydrolysis of SLG in the presence of the thiol group reagent 5-5' dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm (ε = 13.6 mM^{-1} cm^{-1}) (168). Assays are conducted in an appropriate buffer at pH 7.4.

1.9.2 Glyoxalase II – Purification

A typical purification procedure for Glo-II usually begins with fractionation of the cytosolic extract, followed by a variety of chromatography methods (168, 169). Traditionally, fractionation was accomplished by the addition of ammonium sulfate; presently, 70% acetone is more commonly used. Chromatography steps generally include any combination of anion and / or cation exchange, gel filtration, Affi-gel blue

chromatography, hydroxyapatite, and a variety of GSH and GSH derivative affinity chromatography matrices. Until recently, multiple chromatography steps were required to obtain an acceptable level of enzyme purity.

1.9.3 Glyoxalase II – Distribution

Glo-II activity has been found in all prokaryotic and eukaryotic tissues tested to date, and it is therefore considered ubiquitous. Glo-II activity has been detected in a variety of mammals (168,169), a wide variety of vertebrates (170), yeast (171), bacteria (172), helminthes (173, 174), protozoans (91), and fungi (175). It has been purified from a number of sources including human (168), mouse (176) and bovine (177) liver, as well as from plants such as spinach (178), aloe vera (179), and corn (180).

Glo-II activity in mammals is not restricted to any particular tissue although differences in specific activity from tissue to tissue have been reported (see Table III) (37). In humans, the greatest specific activity is found in the brain, with intermediate levels of activity in the liver, kidney, muscle and heart, and very low activity occurring in the spleen and pancreas. In general, mammals exhibit the greatest Glo-II activity in the liver and kidney, and the lowest degree of activity in the spleen. Approximately 15% of the Glo-II activity in a cell is found in the mitochondria, possibly the product of a separate gene (39). The mitochondrial enzyme has been reported to exist in both the intermembrane space and the matrix (39).

1.9.4 Glyoxalase II – Molecular Characteristics

The molecular characteristics of Glo-II from a number of sources are listed in Table IV. Glo-II is isolated as a monomer, although the existence of the enzyme in multimer forms has been reported (181). The molecular weight of the monomeric protein ranges from 21 to 30 kDa, depending on the source.

Isozyme forms of Glo-II have been reported in a number of tissue types from a number of organisms. While only one form of the cytosolic enzyme was reported in both human (168) and murine (176) liver, two forms were reported in rat liver (182). Two forms of the enzyme were reported in human erythrocytes (183) and rat brain (182) as well. In plants, three forms were reported in both aloe (179) and spinach (178), but only one form was detected in corn (180). The mitochondrial enzyme exists in five different forms in the liver of both rat (39) and bovine calf (177), but only one form of the mitochondrial enzyme was detected in spinach (175).

The isoelectric point of Glo-II is also dependent on the source. The values for the mammalian cytosolic enzyme are consistently in the basic region and range from pH values of 7.4 to 8.5 (168, 169, 176, 181, 182). The enzyme from plant (178-180), bacterial (172), and fungal (175) sources exhibit isoelectric points only in the acidic region ranging from a pH of 4 to 6.2. The mitochondrial enzyme from rat (39) and bovine (177) liver exhibit isoelectric points ranging from a pH of 6 to 8.

In the absence of crystallographic data, researchers have turned to methods such as circular dichroic analysis, computer generated sequence analysis, and limited trypsin digestion to gain information on the molecular organization of Glo-II (183). Taken together, the data predict that Glo-II is a typical α / β protein organized in two distinct

domains. The N-terminal region appears to be a highly compact, stable domain consisting almost exclusively of beta-sheet. The active site is thought to be present in this domain. The C-terminal region is predicted to exist almost exclusively as a flexible α-helical domain void of, and non-essential for, catalytic activity.

1.9.5 Glyoxalase II – Substrate Specificity

The substrate specificity of Glo-II from a number of sources is summarized in Table V.

With respect to the GSH moiety of the substrate, Glo-II is highly specific. Neither the α-hydroxyesters of coenzyme A nor those of thioglycolate serve as substrates for the enzyme (168). The enzyme also exhibits no catalytic activity with substrates containing oxygen esters (168). As with Glo-I, the role of GSH in Glo-II activity is thought to be restricted to enzyme recognition.

With respect to the carboxylic acid moiety of the substrate, Glo-II exhibits the ability to accept a broad variety of acids as substrates (168, 184, 185). As with Glo-I, the ability to accept such a broad range of substrates is indicative of other detoxification systems such as the cytochrome P-450 system (130) and GSH-S-transferase enzymes (131). The ability of the enzyme to accept substrates is not limited only to carboxylic acids that possess a hydroxyl moiety on the C2 position. This suggests that Glo-II activity is not restricted to only the products of the Glo-I reaction, but may accept any number of physiological substrates that possess the GSH ester linkage.

1.9.6 Glyoxalase II – Kinetics

The Glo-II reaction appears to follow Michaelis-Menten kinetics over an extensive range of substrate levels using SLG as a substrate (168). SLG exhibits both a relatively low K_M value and the highest Vmax relative to other substrates tested (see Table V) (168, 190). This fact, coupled with the existence of a well-defined source of SLG from the Glo-I reaction, has defined SLG as the physiological substrate for Glo-II. The K_M values of Glo-II for this substrate consistently range from 0.14 – 0.22 mM (see Table III). Exceptions to this are Glo-II derived from yeast, which exhibit K_M values of 7 μM for *Saccharomyces cerevisiae* (171) and 80 μM for *Hansenula mrakii* (172). The cytosolic enzyme from mammals consistently exhibits a specific activity of 600 – 900 μmol / min. / mg (see Table III). Exceptions are values reported for the cytosolic enzymes derived from yeast (1.34 μmol / min. / mg) (171), from corn (65 μmol / min. / mg) (180), and the mitochondrial enzyme from bovine calf liver (134 μmol / min. / mg) (177).

In vitro, quantitative kinetic models have predicted that the glyoxalase system can act in a highly efficient manner in removing methylglyoxal from a cell (186). Contrary to these predictions, the catalytic efficiency of the glyoxalase system *in vivo* has been predicted to be far less than that of the system when studied under such optimal *in vitro* conditions (187, 188).

Quantitative kinetic models have led to the prediction that a purified preparation of Glo-II is an efficient catalyst for the conversion of SLG to GSH and D-lactic acid giving values for k_{cat} / K_M in the $10^5 – 10^6$ range (189). Under conditions in which the rate of hydrolysis of SLG by Glo-II is determined at substrate concentrations $<< K_M$, the

effect of increasing viscosity (using sucrose or glycerol) upon the kinetic parameters of the Glo-II catalyzed reaction resulted in a significant decrease in the value of k_{cat} / K_M (189). This effect was demonstrated to be due to an increase in the K_M for the substrate, with no significant change in k_{cat} being observed (189). From these results, the reaction is believed to be partially limited by diffusion and the rate of formation of GSH and D-lactic acid from SLG by Glo-II was predicted to be as high as 50% of the theoretical limits of diffusion (189). Therefore, the fate of the enzyme – substrate complex is proposed to be distributed equally between the disassociation of the complex to reactants and their conversion to products.

The value of k_{cat} / K_M is also highly dependent upon ionic strength when SLG is used as a substrate (189). Values were found to decrease significantly with increases in ionic strength, an effect determined to be due to an increase in K_M value (189). Therefore, the encounter of substrate with enzyme is likely to be governed to some degree by charge – charge interactions (189).

1.9.7 Glyoxalase II – Active Site Residues

Purified Glo-II from both human liver (168) and rat erythrocytes (184) is rapidly inactivated by the amine reactive reagent, 2,4,6 trinitrobenzenesulphonate. Protection from inactivation by the competitive inhibitor, N-acetyl-S- (p-bromobenzyl) GSH was observed for the enzyme from rat erythrocytes, suggesting the presence of an active site amine.

Purified Glo-II from rat erythrocytes (184), liver and brain (182) is only partially inactivated by the arginine – specific reagent, phenylglyoxal, at pH = 8. Protection from

inactivation in the presence of a competitive inhibitor was observed in all cases. These results suggest the presence of an arginine residue in the active site that is arguably important in substrate binding.

Purified Glo-II from rat erythrocytes (184), liver and brain (182) is rapidly inactivated by the histidine – specific reagent, diethylpyrocarbonate (DEPC) at a pH = 6. With respect to the erythrocyte enzyme, protection from inactivation by a competitive inhibitor was observed but reactivation by hydroxylamine, a reagent known to cleave the DEPC – histidine adduct, could not be achieved (184). With respect to the enzyme from liver or brain, protection from inactivation by the slow substrate, S-mandeloylglutathione, was observed (182). In contrast to the enzyme from rat erythrocytes, reactivation of the enzyme activity by hydroxylamine was achieved (182). These results suggest the presence of a critical active site histidine residue important in catalysis.

Purified Glo-II from rat erythrocytes (184) is not inactivated by the tyrosine - specific reagent, tetranitromethane suggesting that a tyrosine residue is not involved in catalysis. Purified Glo-II from rat erythrocytes (184), liver and brain (182) is also not inactivated by the tryptophan – specific reagent, 2-hydroxy-5-nitrobenzylbromide suggesting that a tryptophan residue is not involved in catalysis. Diisopropylphosphofluoridate or phenylmethylsulfonylfluoride does not inactivate purified Glo-II from rat erythrocytes (184), liver and brain (182). Nor is the enzyme inhibited by classical serine proteinase inhibitors such as leupeptin or chymostatin, suggesting that a serine residue is not involved in catalysis and ruling out the possibility of a serine-dependent thioesterase.

The role of metal ions in Glo-II catalytic activity is unclear. Previously, experiments conducted on Glo-II from human liver (168) suggested that a metal ion is not involved in the catalytic activity of the enzyme. Short-term storage of the enzyme in the presence of chelating agents such as EDTA, EGTA, and 8-hydroxyquinoline had no effect on the activity of the enzyme. On the other hand, long-term storage of the enzyme with EDTA did cause complete labilization of the enzyme, with no reactivation being observed after the addition of a variety of divalent ions. Furthermore, Glo-II activity is unaffected by phosphoamidon, a classical inhibitor of metalloproteinases. Inactivation of the purified enzyme from rat erythrocytes was observed during dialysis against EDTA, 1,10-phenanthroline, or 8-hydroxyquinoline-5-sulfonic acid, but again, no reactivation of Glo-II activity was observed subsequent to the addition of a variety of metal ions (184). These results were interpreted as suggesting that Glo-II is not a metallohydrolase.

Very recently one report suggests that Glo-II isolated from *Arabidopsis thaliana* contains two moles of zinc atom per mole of enzyme (191). It was also reported that this metal ion is required for the activity and / or structural integrity of the enzyme. Comparison of the amino acid sequence of this enzyme, as well as those from humans and yeast has revealed a region of homology with that of the zinc-binding motif found in metallo-β-lactamases. Therefore the involvement of metal ions with respect to Glo-II activity is now a topic of great interest in glyoxalase research. Further study is warranted to determine whether or not the zinc ions are involved directly with catalysis or if the ions maintain the structural integrity of the enzyme, as is the case with Glo-I.

The role of cysteine residues in Glo-II catalytic activity is also unclear. Uotila (168) reported that the enzyme purified from human liver is quite unstable under a variety

of storage conditions, and that activity could be stabilized only by the addition of rather high concentrations of reducing agents such as mercaptoethanol (9.1 mM) and dithiothreitol (5.5 mM). In additional experiments, the enzyme was markedly inhibited in a dose dependent manner by the addition of sulfhydryl group reagents such as $HgCl_2$, HgBzOH, 5-5'-dithiobis(2-nitrobenzoate) (DTNB), iodoacetate, and N-ethylmalemide (NEM). Uotila also reported the partial reactivation of the Hg-modified Glo-II activity by the addition of dithiothreitol. Supplementary studies showed that classical inhibitors of cysteine – dependent hydrolases such as E64 and antipain were ineffective as Glo-II inhibitors (168).

Further studies to determine the role of cysteine residues with respect to Glo-II catalytic activity were conducted upon the purified enzyme from rat erythrocytes (184). In this study, DTNB proved to be ineffective as an inhibitor of Glo-II, contrary to the aforementioned experiments with the human liver enzyme. Incubation of the enzyme in the presence of NEM did cause partial inactivation of the enzyme, although the inhibition reported may have been due to a loss of activity occurring during removal of the reagent from the incubation. No protection from inactivation of enzyme activity was observed during incubation of the enzyme and NEM in the presence of a competitive inhibitor. Further studies on the effect of NEM on the activity of both isoforms of Glo-II from the liver and brain of Wistar rats gave similar results (182).

Taken together, the effect of sulfhydryl group reagents on Glo-II activity all but excludes the possibility of Glo-II being a cysteine-dependent hydrolase. The partial inactivation by NEM may be the result of a modification of a free cysteine residue. Even so, the presence of significant amounts of NEM in a Glo-II assay, a process dependent on

the reactivity of a sulfhydryl group reagent with free GSH, causes interference. Therefore, removal of NEM previous to the assay is required. Such a process may result in loss of activity and therefore misinterpretation of the results. The amino acid sequence of human Glo-II identifies six cysteine residues, all of which are located in the N-terminal region where the active site has been predicted to be located. To date, no information on the free sulfhydryl content of any form of Glo-II has been reported.

1.9.8 Glyoxalase II – Catalytic Mechanism

Results of the aforementioned chemical modification and inhibitor studies exclude the possibility of Glo-II being a serine-dependent or cysteine dependent hydrolase. The assumption that a metal ion played no role in the hydrolytic activity of Glo-II rules out the possibility of Glo-II being a metallohydrolase as well. A general acid – general base mechanism of catalysis was also ruled out since studies with isotopic water showed only a very small effect of the solvent isotope (184). An E1cB mechanism was ruled out as well, since S-α-deuterated substrates catalyzed by Glo-II did not exhibit any loss of the deuterium by the products of the reaction (184).

Studies that identified an active site histidine in the enzyme that is essential for catalysis, coupled with results from the aforementioned studies, have led to a proposed histidine-dependent mechanism of Glo-II catalysis (184). The mechanism proposes the direct nucleophilic attack of the active site histidine on the thioester substrate. This proposal is supported by the known susceptibility of thioesters to aminolysis (192). Predictably, the reaction would then proceed via the formation of an acyl-imidazole intermediate, freeing GSH as a result of the reaction. The acyl-imidazole intermediate

would then undergo rapid hydrolysis resulting in the release of the hydroxyacid to the solvent. This is consistent with the known lability of acyl-imidazoles (192).

Although researchers for a number of years accepted the proposal for the catalytic mechanism of Glo-II, recent advances in glyoxalase research have raised doubts to its validity. Recent reports have identified the presence of two moles of zinc per mole of Glo-II enzyme purified from *Arabidopsis thaliana* (191). Comparison of the amino acid sequence of this enzyme with those from humans and yeast has revealed a region of homology with that of the zinc-binding motif found in metallo-β-lactamases. Research is ongoing to define the role of zinc with respect to Glo-II catalytic mechanism and / or structural integrity. The catalytic mechanism of metallo-β-lactamases is known to proceed through an acyl-intermediate as well, although the residue acylated in the lactamase reaction is a serine residue (193) while the proposed acylated residue in Glo-II is histidine.

1.9.9 Glyoxalase II - Inhibitors

To date, the only natural metabolites that are known to inhibit Glo-II activity are GSH (168) and the methylglyoxal – derived hemithioacetal substrate for Glo-I (24, 140, 171, 176). The inhibition by GSH is quite weak, with the K_I in the high millimolar range (168). The concentration of the hemithioacetal needed to provide a significant level of Glo-II inhibition (Ki = 100-200 μM) is far above the predicted physiological concentration of the hemithioacetal (194). Therefore, the inhibition of Glo-II by these compounds is not considered to be physiologically relevant.

A number of substrate analog inhibitors derived from GSH have been synthesized. Among the most potent and specific of those inhibitors are thiocarbonate derivatives and thiocarbonate / carbamate derivatives of GSH (180, 195-199). These inhibitors generally exhibit a high degree of specificity for Glo-II with Ki values in the low micromolar range. Table VI summarizes a number of Glo-II inhibitors developed to date.

1.9.10 Glyoxalase II – Genetics and Cloning

In humans, the gene for Glo-II, HAGH, is located on chromosome 16, region 16p13.3 (200, 201). Although humans are reported to express two forms of cytosolic Glo-II, polymorphism is rare and has been reported only in Micronesian (200) and Japanese (201) populations. The more commonly expressed form of Glo-II, HAGH1, is known to exist in two isoforms which may be separated by non-denaturing gel electrophoresis (167).

A DNA fragment encoding Glo-II has been isolated from a human adult liver cDNA library (202). This DNA fragment consists of 1011 base pairs and a full-length coding region of 780 base pairs, corresponding to a protein with a calculated molecular mass of 28,861 daltons (202). A vector was successfully constructed for heterologous expression of Glo-II in *E. coli.*

A cDNA encoding Glo-II from *Arabidopsis thaliana* has also been cloned and sequenced (203). It consisted of 894 base pairs and a full-length coding region of 774 base pairs, corresponding to a protein with a calculated molecular mass of 28,791 daltons

(203). A vector was also successfully constructed for the heterologous expression of Glo-II in *E. coli*.

Analysis of a T-DNA-tagged mutant of *Arabidopsis thaliana* has identified the gene for, and has led to the isolation of, the cDNA of a Glo-II isozyme that appears to be mitochondrially localized. The cDNA encoding a Glo-II cytoplasmic isozyme was also isolated and characterized. Southern blot and sequence analyses indicate that the Glo-II proteins are encoded by at least two multigene families in *Arabidopsis thaliana*. *E. coli* cells expressing either form of the enzyme exhibit increased Glo-II activity, confirming that they do encode Glo-II proteins. Northern analysis shows that the two genes are differentially expressed, in that transcripts for the mitochondrial isozyme are most abundant in roots, while those for the cytoplasmic isozyme are highest in flower buds. The identification of Glo-II isozymes that are differentially expressed suggests that they may play different roles in the cell.

Strong sequence homologies (50-60%) to the human liver Glo-II were found within a limited region of GSH transferase I cDNA from corn (202). The amino acid sequence deduced from the *A. thaliana* cDNA showed 54% identity with that of the human enzyme (203). Databank searches identified seven additional DNA sequences from different species with high similarity to Glo-II. Certain limited regions, one rich in histidine residues, shared 100% identity (203). Such data suggests that Glo-II from different species may originate from a common ancestor and that other GSH requiring enzymes may be related as well.

1.10 Glyoxalase Activity - Proliferation, Differentiation, and Activation of Cells

Normal development in differentiating fetal tissues has been correlated with an increased capacity to metabolize methylglyoxal. It is accepted that the glyoxalase phenotype is one of the earliest developed in fetal tissues. In humans, the ability to metabolize methylglyoxal exhibits full development before the end of the sixth week of gestation and subsequently increases in activity until birth (205). The increased ability to metabolize methylglyoxal has been demonstrated to be due to a three-fold increase in Glo-I activity in comparison to corresponding fully developed adult tissue.

Studies on developing chick embryos produced results similar to those obtained in human fetal studies (206). The livers of embryonic chicks exhibit a high level of Glo-I activity but comparably low Glo-II activity during the process of differentiation before hatching. Furthermore, Glo-I activity decreased, while Glo-II activity increased, after hatching. The changes in glyoxalase activity under these conditions were not due to the appearance of different isozymes during development.

The activities of the glyoxalase enzymes undergo changes during aging as well. Studies on tissues from aged rats' (214) and mice (105) were conducted to determine if any changes in glyoxalase enzyme activity occurred in tissues due to aging. In these cases, the levels of both glyoxalase enzymes were greatly reduced in most tissues studied relative to corresponding tissues from younger subjects.

Other studies have been conducted on regenerating liver tissue to determine the status of the glyoxalase system in rapidly proliferating tissues. Regeneration of liver tissue has been induced by partial hepatectomy (104), radiation damage (208), or the addition of carcinogens to the diet; conditions that result in an uncontrolled state of

proliferation (209). In all cases, a significant increase in the activity of Glo-I and a correlative decrease in the activity of Glo-II occurred. Other studies in which the glyoxalase activity of rapidly proliferating tumor cells were compared to that of normal corresponding tissues (210, 211) demonstrated high Glo-I activity, whereas Glo-II activity was either undetectable or at least greatly reduced.

The activation of resting blood cells such as platelets causes significant changes in the ability of these cells to metabolize methylglyoxal. Resting platelets completely metabolize methylglyoxal to lactate, while stimulated platelets transform only 10 – 15 % of the α-ketoaldehyde to lactate (212). The stimulation of these cells by agonists, in particular thrombin, was later demonstrated to result in the accumulation of SLG in a dose dependent manner (213), suggesting the inhibition of the Glo-II reaction. The addition of methylglyoxal to platelets *in vitro* has been demonstrated to produce an oxidative effect resulting in an increase in hydrogen peroxide, an increase that is potentiated by thrombin stimulation (212). Furthermore, the addition of methylglyoxal to platelets in vitro inhibits both the agonist-induced aggregation of platelets as well as the release of ATP from platelets (212).

Further studies have also been conducted on the status of the glyoxalase system during the functional activation of both human polymorphonuclear leukocytes (22) and resting neutrophils (72, 73). Both human polymorphonuclear leukocytes and resting neutrophils, when stimulated by phorbol ester treatment, in culture, demonstrated a significant increase in Glo-I activity. This increase was proposed to be due to a significant increase in the maximum velocity of the enzyme (187). Glo-II activity decreased after stimulation, and was proposed to be due to a significant decrease in the

maximum velocity of the enzyme (187). Since the Michaelis constants for both enzymes did not undergo any change during stimulation, it was suggested that the stimulatory process caused the appearance of an unidentified non-competitive binding constituent that affected glyoxalase activity.

Studies have been conducted on the status of the glyoxalase system in differentiating blood cells (207). Both human promeolytic leukemia (HL60) cells and erythroleukemia cells in culture exhibited high Glo-I activity and low Glo-II activity prior to differentiation. The chemically induced differentiation of these cells resulted in a significant loss of Glo-I activity; this was later shown to be due to a significant decrease in the maximum velocity of the Glo-I activity (187). These cells also exhibited an increase in Glo-II activity after differentiation, suggesting (to the investigators) a significant increase in the maximum velocity of the Glo-II activity of the cytosolic extract (187). Since the Michaelis constants for both enzymes under these conditions did not undergo any change during differentiation, results were again related to the existence of an unidentified non-competitive binding constituent that affected glyoxalase activity. The decrease in the ratio of Glo-I / Glo-II activity in differentiating blood cells is in contrast to the increase in the ratio of Glo-I / Glo-II activity in blood cells which have been by activated from the resting state by chemically - induced stimulation.

Overall, glyoxalase activity appears to undergo changes as the physiological state of the cell changes with respect to proliferation, differentiation, and activation. Rapidly proliferating cells appear to exhibit a consistent and significant increase in the ratio of Glo-I / Glo-II activity when compared to corresponding quiescent tissues. Upon activation, blood cells such as platelets, leukocytes, and neutrophils appear to undergo

similar increases in the Glo-I / Glo-II ratio when compared to corresponding cells in a resting state. In contrast, differentiating blood cells appear to exhibit a decrease in the ratio of Glo-I / Glo-II activity. Limited evidence has led to the proposal of the existence of a metabolite that affects glyoxalase activity in a non-competitive manner.

1.11 Glyoxalase Activity – Relationship to Glucose Metabolism

Although any direct relationship between the glyoxalase system and the glycolytic pathway had been discounted, flux through the glyoxalase pathway as well as the fate of glyoxalase metabolites appears to be indirectly affected by the state of glucose metabolism in an organism.

Studies involving the stimulation of glucose metabolism by way of anaerobic exercise in humans demonstrated a decrease in methylglyoxal concentrations with a corresponding increase in D-lactate levels in both plasma and red blood cells (81). These results were interpreted as indicating that an increase in glucose metabolism stimulates an increase in the flux through the glyoxalase pathway. Since the levels of D-lactate in excretory fluids (urine and sweat) were unchanged, it was assumed that the body in some other process utilized the D-lactate.

In other studies designed to determine the relationship of the glyoxalase system to glucose metabolism, methylglyoxal was shown to be a gluconeogenic precursor in rats who had been starved under a variety of conditions (215), a process that had been later suggested to be calcium dependent (216). It is possible that methylglyoxal could enter the gluconeogenic pathway at the position of pyruvate, either by the oxidation of methylglyoxal directly to pyruvate by methylglyoxal dehydrogenase (E.C. 1.2.1.23)

(48, 49), or by the conversion of D-lactate to pyruvate by 2-hydroxyacid dehydrogenase (79). Even so, the inhibition of glyceraldehyde-3-phosphate dehydrogenase did not affect the flux of methylglyoxal to glucose in isolated murine hepatocytes; this was interpreted as indicating the existence of an alternative pathway from methylglyoxal to glucose (217).

Studies involving the fate of methylglyoxal in human diabetic subjects also indicate that the flux through the glyoxalase pathway appears to be indirectly affected by the state of glucose metabolism in an organism. Isolated red blood cells from normal humans cultured under hyperglycemic conditions demonstrated an increased flux of methylglyoxal to D-lactate with no detectable corresponding changes in enzyme activity (218). In another study, the levels of methylglyoxal, SLG, and D-lactate were all demonstrated to increase in the blood samples of diabetic patients, indicating an increase in flux through the pathway (219).

Studies comparing normal rats to streptozotocin-induced diabetic rats (a model for insulin dependent diabetes) have provided further support that flux through the glyoxalase pathway is affected by the state of glucose metabolism in an organism (82, 220). Glycolytic tissues (lens and blood) demonstrated increased concentrations of both methylglyoxal and D-lactate in diabetic rats when compared to controls (82). Insulin dependent tissue (skeletal muscle) exhibited a decrease in methylglyoxal concentration with a corresponding increase in the concentration of D-lactate in diabetic rats when compared to controls (82). In gluconeogenic tissue (liver), the concentration of methylglyoxal was also decreased with a corresponding increase in the concentration of D-lactate (82). In another study involving the same model, glyoxalase enzyme activity

increased in its capacity to metabolize methylglyoxal in skeletal muscle (220). In contrast to skeletal muscle, glyoxalase enzyme activity in the liver was decreased in its capacity to metabolize methylglyoxal (220).

Studies using the obese diabetic rat (a model for non-insulin dependent diabetes) have provided further support that flux through the glyoxalase pathway is affected by the state of glucose metabolism in an organism (82). An overall increase in the conversion of methylglyoxal to D-lactate was demonstrated in the tissues of these subjects. Furthermore, the concentration of methylglyoxal increased in blood samples, but also decreased in both muscle and liver. These results parallel those in studies on the streptozotocin-induced diabetic rat.

It should be noted that glycerol was demonstrated to be the best substrate tested as a carbon source for the formation of D-lactate, followed by glucose, in both the cytosolic fraction of rat liver as well as that of perfused rat liver (221). It is well documented that the levels of both glycerol kinase (82) and hepatic glycerol (221) increase dramatically under diabetic conditions in these same subjects.

Overall, evidence indicates that the flux through the glyoxalase system is indirectly affected by the state of glucose metabolism in an organism. Predictably, in an organism where a normally functioning glyoxalase system is operating, the steady state levels of methylglyoxal found in tissues are predictably too low to cause significant damage to an organism. Therefore, damage due to toxic methylglyoxal may occur only when the physiological balance of the organism is disrupted, resulting in a chronic increase in the levels of toxins such as methylglyoxal. Diabetes is a plausible example of such disruption and the effect of chronic increases in the level of methylglyoxal in

diabetic subjects has become the topic of increased interest. Current research topics include the formation of advanced glycosylation endproducts (AGE proteins) (66-68) and the development of diabetic complications such as cataract formation (65), as well as the modification of connective tissue proteins (222).

1.12 Background to the Present Work

1.12.1 Rate of Flux through the Glyoxalase System

As stated earlier, changes in either the proliferative state (in particular cancerous tissue) or changes in the state of glucose metabolism (in particular in diabetic patients) in any given tissue also causes changes in the flux of methylglyoxal to D-lactate through the glyoxalase system. It is due to these findings that a renewed interest in the glyoxalase system has occurred. This system is regarded as a possible target in both anticancer therapies and the alleviation of diabetic complications. Therefore, studies that provide a clear understanding of the mechanism by which the glyoxalase system undergoes such changes in flux is, obviously, well timed.

Although the activity of the glyoxalase enzymes has been demonstrated to undergo changes under the aforementioned conditions, no definitive evidence for allosteric control or control of flux by way of induction of the glyoxalase enzymes exists to date. Researchers have therefore begun to rely upon the comparison of data derived from quantitative kinetic models to that of *in vivo* experiments to develop a more clear understanding of the factors that may control the kinetic properties of the glyoxalase system inside cells.

In order to ascertain the efficiency of the glyoxalase system in the removal of methylglyoxal from cells, as well as determine if any unknown kinetic factors are involved in the conversion of methylglyoxal to D-lactate, quantitative models of the pathway have been developed (186, 189, 223). To date, all studies indicate that the steady state concentrations of the substrates for the glyoxalase enzymes *in vivo* are maintained at subsaturating levels ($<< K_M$ values reported for that enzyme) (110, 186, 187, 189, 223). Therefore, results from *in vitro* studies have been used to propose a model for the metabolic flux through the system under conditions in which substrate concentrations are much lower than the K_M values for the two enzymes (186, 189, 223).

Under these conditions, the overall rate of the conversion of methylglyoxal to D-lactic acid is thought to be limited by the rate of formation of the hemithioacetal substrate for Glo-I (186). This interpretation was obtained from calculations based upon free energy diagrams derived from apparent rate constants for each of the reactions that occur within the glyoxalase system (186). The concentration of GSH in tissues is generally maintained at concentrations high enough to be kinetically unimportant in the reaction (186). Therefore, the formation of the hemithioacetal is then predicted to be dependent upon the concentration of free methylglyoxal; the flux through the system is thus under substrate level control.

Both of the glyoxalase enzymes in their purified state appear to act at near optimal catalytic efficiency (189, 223). Values for k_{cat} / K_M ($3.5 * 10^6$ M^{-1} s^{-1} for yeast Glo-I, $8.8 * 10^5$ M^{-1} s^{-1} for rat erythrocyte Glo-II, at pH 7, 25°C) approach the estimated upper limits for such values (224). By the application of the viscogenic variation method to kinetic studies, both enzymes have been demonstrated

to be approximately 50% limited by the rate constant for the diffusion controlled encounter between the active site of the enzyme and the substrate (k_1) under conditions where substrate concentrations are << K_M (189, 223). Thus, in the purified state, both of the glyoxalase enzymes are significantly diffusion-limited and therefore seem to have evolved to a high level of catalytic efficiency.

Whether or not the activities of the glyoxalase enzymes are diffusion limited inside the cell is still in question. If the quantitative kinetic model of the glyoxalase pathway is correct, the concentration of the pathway intermediates can be estimated from the net production of D-lactate in any given tissue and the intracellular concentrations of the glyoxalase enzymes (223). Using this kinetic model, the steady state concentrations of the intermediates were estimated to be 0.7 ± 0.1 nm for the methylglyoxal-derived hemithioacetal and 1.9 ± 0.5 nM for SLG (223).

Contrary to these estimates, the concentrations of the intermediates of the glyoxalase system in human erythrocytes have been reported as exceeding the calculated steady state concentrations by orders of magnitude (0.8 ± 0.14 μM for the hemithioacetal, 14.9 ± 4.6 μM for SLG) (110, 187). The difference between the estimated concentrations of the pathway intermediates in kinetic models and tissue studies led to the proposal of the existence of an as yet unidentified inhibitor of glyoxalase activity, resulting in the unexpected high steady state concentrations of the pathway intermediates (110, 187, 223). This proposal was further supported by experiments that detected approximately 10^4 – fold increases in Glo-II activity in red blood cells upon cell lysis (110). In those cases studied to date, the intracellular activities of other non-allosteric, water-soluble

enzymes, the activities are unchanged upon cell lysis (225, 226). Further, no effective physiological inhibitor of glyoxalase activity has been identified.

Faced with this discrepancy, researchers have turned to alternative explanations for the unexpected high concentrations of glyoxalase pathway intermediates. The discrepancies may be, in part, attributed to the reliability of the assay systems used. Thus, the increase in methylglyoxal / hemithioacetal concentrations could be, in part, due to aberrant sources of methylglyoxal arising from the buffer-ion catalyzed decomposition of trioses and triosephosphates in biological extracts (32). This proposal does not explain the higher than expected intracellular concentrations of SLG. Only a small percentage of the discrepancy in these estimates has been demonstrated to result from either the acid-catalyzed esterification of GSH by L-lactate during the acid denaturing of whole blood samples (227) or the overestimation of SLG concentrations due to the presence of both the D and L isomers of the intermediate (228).

A second explanation for the discrepancies in the concentrations of glyoxalase pathway intermediates is that the small steady state concentrations estimated from the quantitative models are in equilibrium with much larger pools of these intermediates that are reversibly bound to intracellular proteins (223). The higher methylglyoxal / hemithioacetal concentrations has been demonstrated to be, in part, due to the reversible binding of methylglyoxal to proteins, particularly cysteine, arginine, and lysine residues (229). Again, this does not explain the increase in intracellular concentrations of SLG. To date, no evidence for the existence of an intracellular pool of reversibly bound SLG exists. Therefore, although the unexpected increases in the intracellular concentrations of

the methylglyoxal / hemithioacetal intermediates in cells may have been accounted for, no explanation for the high concentrations of the substrate for Glo-II, SLG, exists to date.

1.13.2 Inhibition of Glo-II Activity by Purine Nucleotides

Some years ago, it was observed in this laboratory that the Glo-II activity of a crude cytosolic extract of rat liver is significantly inhibited when assayed in the presence of ATP or GTP (0.3 – 1.0 mM). Recently, we have conducted a limited nucleotide inhibition survey of plant and bacterial cell free extracts, as well as the cell free extracts from various organs from a number of animal species. We have found that ATP and GTP typically inhibit the Glo-II thiohydrolase activity of those cytosolic extracts from plant and animal species. On the other hand, ATP or GTP did not significantly inhibit Glo-II activity in cytosolic extracts of *Escherichia coli*. Table VII summarizes these results.

Although the inhibition of Glo-II activity by ATP or GTP is observed in the cell free extracts of all higher animals and plants tested, it was also found that Glo-II, purified by traditional methodologies, is no longer inhibited by ATP or GTP. The loss of nucleotide sensitivity during the purification process was at first thought to be due to the removal of some unidentified factor during the purification. Since studies to identify such a factor were unsuccessful, it became apparent that the sensitivity of thioesterase activity to ATP or GTP must be an intrinsic characteristic of the enzyme itself.

1.12.2 Hypothesis and Objectives

The central hypothesis of this work is that Glo-II can exist in two forms, one of which is sensitive to inhibition by certain nucleotides while the other is not. The purpose of this work is to provide the tools needed for the purification and characterization of each of these forms of Glo-II.

Objective 1; Purification of Glo-II-s

Studies were initiated to determine if an enzyme purification procedure could be developed such that the pure enzyme retains its sensitivity to inhibition by nucleotides. Purification processes reported in literature for the preparation of homogenous Glo-II employ several fractionation steps, and require days to complete (168, 169). ATP or GTP does not significantly inhibit pure preparations of Glo-II obtained by such procedures.

As the following objectives were dependent upon the success of the first objective, in our earlier studies we had developed a rapid, one-step affinity chromatography method to purify bovine liver Glo-II from cytosolic fractions. The Glo-II activity purified under these conditions is significantly inhibited when assayed in the presence of ATP or GTP. For convenience, we have designated Glo-II activity that is significantly inhibited by physiological levels of ATP as “sensitive Glo-II” (Glo-II-s). We have designated Glo-II activity that is not significantly inhibited by physiological levels of ATP as “insensitive Glo-II” (Glo-II-i). Only one reference, originating from work in our laboratory, refers to the inhibition of Glo-II activity by nucleotides (25).

Objective 2; Determination of the inhibition properties of Glo-II-s.

In order to set the experimental parameters to be used in subsequent studies, the stability of Glo-II-s with respect to temperature and pH were to be determined. Once this step was completed, a survey was to be conducted to determine which nucleotides inhibit Glo-II-s, and of these, which ones are most inhibitory. Once these investigations were completed, the kinetic parameters of Glo-II-s and the type of inhibition by nucleotides were to be determined.

Objective 3; Investigation of the conditions which result in the loss of nucleotide inhibition by Glo-II-s

In our initial studies, we have observed the gradual loss of nucleotide sensitivity by Glo-II-s after purification. Standardized conditions, which cause the loss of nucleotide sensitivity by Glo-II-s in a reasonable time period, were to be determined. Such conditions would allow for a repeatable laboratory process for the acquisition of the resultant Glo-II-i for further study.

In other early studies, we have observed the rapid loss of nucleotide sensitivity of Glo-II-s by short-term incubation with certain inhibitory nucleotides. Further investigations of this observation were also to be conducted in order to determine if these conditions also cause the transition from Glo-II-s to Glo-II-i.

Objective 4; Determination of the kinetic properties of Glo-II-i.

Once standardized, repeatable conditions for the transition of Glo-II-s to Glo-II-i were found, the kinetic parameters of Glo-II-i were to be determined and compared to those of Glo-II-s.

Objective 5 – Investigation of the structural / conformational differences between Glo-II-s and Glo-II-i.

A number of biochemical studies were to be conducted in order to investigate any structural / conformational differences in the protein that occur upon the transition of Glo-II-s to Glo-II-i. Studies were to determine if the transition is due to:

1. a change in the molecular weight of the enzyme (as determined by SDS-PAGE),
2. a change in the molecular shape (conformation) of the enzyme, or the formation of multimer forms of Glo-II (as determined by gel-filtration), and
3. a change in the structural conformation of the enzyme (as determined by circular dichroic analysis and / or tryptophan fluorescence).

Objective 6; Chemical modification studies on Glo-II-s

A series of chemical modification studies were to be conducted on samples of Glo-II-s in order to investigate the effect of modification on both the activity and inhibition of Glo-II activity by nucleotides. Results from this study will be compared to results from previous chemical modification studies on Glo-II preparations previously reported in literature.

CHAPTER II

METHODS

Materials

Affi-gel-10 affinity chromatography material was purchased from Bio-Rad. DTA was purchased from U.S. Biochemical. All other chemicals were purchased from Sigma. All solvents used were of reagent grade.

Methods

Synthesis of Affi-gel 10-glutathione (Af-SG) Affinity Chromatography Matrix

The Af-SG material, in which the sulfhydryl group links the GSH to the matrix, was synthesized by the method of Norton et al (180) with modifications. First, GSH, 350 mg, was dissolved in 50 mL of ice cold sodium acetate, 30 mM, pH 5.5. The pH was then adjusted to 5.0 with a 10% sodium hydroxide solution. Second, a wet volume of 25 mL of Affi-gel 10 was washed with 5 volumes of ice cold sodium acetate buffer, 30 mM, pH 5.5. Third, the washed Affigel –10 was added to the GSH solution and gently shaken overnight at 4°C. Finally, the resulting solid Af-SG material was washed with 10 volumes of 50 mM TRIS buffer, pH 7.4 to remove excess GSH. The resulting suspension was utilized in the affinity chromatography step in the purification of Glo-II.

Abbreviations: SLG: s-D-lactoylglutathione, GSH: glutathione, DiFMOC-G: N,S-bisflourenylmethoxycarbonyl-glutathione, DEPC: diethylpyrocarbonate, EDTA: ethylenediamine tetraacetic acid, NEM: N-ethylmalimide, DMSO: dimethylsulfoxide, PMSF: phenylmethylsulfonyl fluoride, DTT: dithiothreitol, GSSG: glutathione (oxidized), DTA: meso-2,5-dimercapto-N,N,N',N'-tetramethly-adipamide, ApppA: diadenosine triphosphate, PolyA: polyadenylic acid, DTNB: dithiobis-nitrobenzoic acid, EGTA: ethylene glycol-*bis* (β-aminoethyl ether)N,N,N',N'-tetraacetic acid, TRIS: tris[hydroxymethyl]aminomethane, TES: N-trsi[hydroxymethyl]methyl-2-aminomethanesulfonic acid.

Preparation of Di-FMOC-G Solution

The Di-FMOC-G solution was prepared by dissolving 10 mg DiFMOC-G, sodium salt, in 0.5 mL of DMSO with vigorous shaking. The resulting solution was then added to 9.5 mL of 100 mM TRIS, pH 7.4, at room temperature with vigorous shaking. After cooling to 5°C, any turbidity was removed using a 0.45 μm filter.

Preparation of the Cytosolic Fraction from Calf Liver Tissue Homogenate

Livers were obtained from healthy bovine calves at the time of death and stored in saline ice water slurry during an average transport period of 1 hr.. All further steps were carried out without delay at 0-4°C with 100 mM TES buffer, pH 7.4 (buffer A) unless otherwise stated. The tissue was homogenized with 1 volume of buffer A, containing 100 μM PMSF, for 1 min. using a household blender at low speed. To this homogenate was added a solution of two volumes of buffer A and 1 volume of glycerol and the resulting mixture was blended at high speed for two, 1 min. intervals. The homogenate was then filtered through sterile gauze and centrifuged at 14,000 x g for 1 hour at 5°C. The pellet was discarded. The supernatant solution was recovered and used in further purification steps.

Acetone Fractionation of the Cytosolic Fraction from Calf Liver Tissue Homogenate

Three volumes of cold acetone (-20°C) were added slowly to the cytosolic fraction from calf liver tissue homogenate with gentle mixing and stirred for 30 min. at 1°C. The solution was then centrifuged at 14,000 x g for 10 min. at 5°C. The supernatant solution was discarded. The pellet was dissolved into one volume of buffer

A by gentle stirring for 1 hr. at 4°C and then centrifuged at 14,000 x g, 5°C, for 2 hr.. The resulting pellet was discarded. The supernatant solution (acetone fraction) was recovered and used in further purification steps.

Purification of Glo-II by Af-SG Affinity Chromatography

All steps were conducted at 4°C at a pH of 7.4 unless otherwise stated. GSH-Affigel-10 was added to a small column (1 x 1 cm. gel bed volume), and equilibrated with 100 mL buffer A. The acetone fraction (200 mL) was passed through the column, at a flow rate of approximately 100 mL / hour. Fractions (0.25 ml) were collected from the column and assayed periodically until saturation of the column had occurred. Non-specifically bound protein was then removed from the column by washing with 100 mL of buffer A, followed by 50 mL of 150 mM TES, pH 7.4, followed by 10 mL of 200 mM TES, pH 7.4, and finally 10 mL of buffer A.

Elution of the enzyme was carried out with a 0.5 mM solution of DiFMOC-G, 10 mL, at a flow rate of 5 mL/min. Typically, elution was complete with the application of about 5 mL of elutant. The eluate obtained from the column was incubated at 5°C for 1 hr. to allow for hydrolysis of the Di-FMOC-G by the Glo-II activity. Then, four volumes of cold acetone (-20°C) were added, and the solution was incubated at -20°C for 5 hr. The sample was then centrifuged at 16000 g, 5°C, for 1 hr.. The supernatant solution was removed and the pellet (pure Glo-II) was dissolved in 1 mL of buffer A. The resulting sample was typically apportioned into 20 Eppendorf tubes (50 uL / tube), and stored at 80°C until use.

Protein Determination of Purified Glo-II Preparations

Protein determinations were carried out according to the method of Bradford (230) using a protein determination kit purchased from Sigma, bovine serum albumin as a standard, and procedures as set by the manufacturer.

Determination of Molecular Weight and Analysis of Purity of Glo-II Preparations

Analysis of the purity of Glo-II preparations was carried out by sodium dodecyl–sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (231). Electrophoresis was conducted using a Bio-Rad mini-gel apparatus, with a 3% acrylamide stacking gel and a 15% acrylamide resolving gel, according to the manufacturer's specifications. Determination of the molecular weight of the enzyme was done by comparison of the relative mobility of the protein to those of a set of low molecular weight standards purchased from Sigma and treated according to the manufacturers specifications. Detection of the protein was conducted by utilizing both Coomassie blue (232) and silver staining (233).

Synthesis and Standardization of SLG

SLG was prepared and purified according to the method of Uotila (234). Stock solutions of SLG (50 mM) were made up in distilled water and their concentrations were standardized by enzymatic endpoint assay (extinction coefficient = 13600 M^{-1} cm^{-1} at 412 nm) which involves the hydrolysis of SLG to D-lactate and reduced GSH in the presence of DTNB when catalyzed by the addition of Glo-II.

Standardization of Nucleotide Solutions

Nucleotide stock solutions were made up in distilled water and their concentrations were determined by UV spectroscopy using previously published extinction coefficients (235).

Routine Glo-II Assay

Glo-II activity was measured by following the increase in absorbance at 412 nm (extinction coefficient = 13.6 mM^{-1} cm^{-1}) due to formation of the chromophoric adduct between 5-5'dithiobis(2-nitrobenzoic acid) (DTNB) and the free, reduced GSH released from the enzymatic hydrolysis of the thioester, SLG. A typical assay mixture contained 0.2 mM DTNB, 0.25 mM SLG, and 0.01 to 0.04 units of enzyme solution per mL of assay mixture in buffer A, at 25°C. The reaction, which was initiated by the addition of enzyme, unless otherwise stated, was followed for 1.5 min. on a Shimadzu UV-VIS spectrophotometer, model 1201, equipped with a kinetic software program. Initial rates were determined from the slope of the linear plot generated by the increasing absorbance from the chromophore formation. A blank without enzyme was always included. One enzyme unit is defined as the amount of enzyme (Glo-II) required to convert 1 μmol of SLG to GSH and D-lactate at 25°C per minute under the assay conditions described.

Temperature Stability Studies on Glo-II-s

Aliquots of purified Glo-II-s (2.5 U / mL) were incubated at various temperatures and aliquots were removed, diluted, and assayed periodically for activity. All assays were conducted as described above at a SLG concentration of 0.25 mM.

Studies of the Effect of pH on the Stability of Purified Glo-II-s

Aliquots of Glo-II-s (2.5 U / mL) were incubated at various pH values for five min. on ice. The incubation buffer used contained 40 mM citrate, 40 mM TES, 40 mM TRIS, adjusted to specific pH values ranging from 4-10. Aliquots of the enzyme incubation were removed at designated time intervals, diluted 20-fold into buffer A, and assayed for activity in the usual assay buffer. Data were recorded as the percent activity relative to a fresh, 20-fold dilution of the enzyme in which the pH had not been altered. The SLG concentration in the assay was 0.25 mM.

Studies of the Effect of pH on Glo-II-s, Activity and Nucleotide Inhibition

Glo-II-s was incubated at a variety of pH values (5.5-8.5) in a buffer containing 40 mM citrate, 40 mM TES, 40 mM TRIS, 0.4 mM SLG for 1 minute at 25°C in a final volume of 1.0 mL. The reaction was started by the addition of substrate. The reaction was then quenched by the addition of 300 uL of 1N HCl. After a five minute incubation under these conditions, to ensure irreversible enzyme denaturation, the pH of the solutions were brought to pH 7.4 by the addition of 2.7 mL of a 1 M TES buffer at pH 7.4, containing 0.8 mM DTNB. For all samples a blank with no enzyme present was treated similarly. The absorbance of each sample was then read at 412 nm against the appropriate blank. The experiments were also conducted in the presence of 2 mM ATP.

Inhibition Studies with Nucleotides

Glo-II-s was assayed in the presence of various nucleotides to determine which of them were inhibitory. The nucleotides tested included ATP, ADP, AMP, GTP, GDP, GMP, CTP, UTP, ITP, IMP, NAD, NADH, NADP, NADPH, FMN, pppGpp, ppGpp, ApppA, ADPR, and PolyA. Nucleotide solutions were prepared as previously described and the final concentration in the assay was 2 mM. The assays were also conducted as previously described at a SLG concentration of 0.25 mM.

Incubation Studies with Nucleotides

Nucleotides tested were ATP, ADP, AMP, GTP, GDP, GMP, and UTP. Samples of concentrated Glo-II-s (20 U.) were incubated for 1 hour at 12^{O}C in the presence of nucleotide (1.4 mM). Samples were then diluted (1:500) into the assay medium such that residual nucleotide concentration from the incubation was 2.8 μM (a non-inhibitory concentration). All samples were then assayed as previously described at a SLG concentration of 0.25 mM. A control, in which no nucleotide had been added, was also included.

Kinetic and Inhibition Studies

Lineweaver-Burk plots were constructed from the experimental data. Final SLG concentrations in the kinetic assays ranged from 0.075 – 1.000 mM. Final nucleotide concentrations in the kinetic studies ranged from 0.0 –1.0 mM. Graphical representation of the data was completed using a Microsoft Excel software program, Version 8.0a. Lines were drawn using linear regression. Replots of the calculated data were

constructed from the corresponding Lineweaver-Burk data and lines were drawn using either linear or polynomial regression programs.

In assays designed to determine the percent inhibition of Glo-II by ATP, the SLG concentration in all assays was 0.25 mM. ATP concentrations in the assays ranged from 0-10 mM. The values of the percent inhibition of Glo-II activity at constant SLG concentration and increasing ATP concentrations were calculated from the ratio of the inhibited rate to the uninhibited rate. Graphical representation of the percent inhibition data was completed using a Microsoft Excel, Version 8.0a program. Lines were drawn using polynomial regression.

Conversion of Glo-II-s to Glo-II-i at 12°C

Glo-II-s (2.5 U / mL) was incubated at 12^0C and aliquots were removed at designated time intervals, diluted, and assayed for activity. Assays were conducted in the absence and in the presence of 2 mM ATP as earlier described. The SLG concentration in the assay was 0.25 mM. Data were recorded as the percent residual Glo-II activity and as the percent residual inhibition of Glo-II by ATP, as compared to initial values (zero time incubation).

Conversion of Glo-II-s to Glo-II-i by Incubation with ATP

Samples of concentrated Glo-II-s (20 U / mL) were incubated for 2 hr. at 1^OC in the presence of 5 mM ATP. At designated times during the 2 hour period, aliquots were removed and diluted into the assay medium such that the residual nucleotide concentration in the incubation mixture was 6 μM (a non-inhibitory concentration).

Controls with no ATP present were treated similarly. After dilution, samples were assayed at 0.25 mM SLG to determine any changes in activity. Samples were also assayed at 0.25 mM SLG in the presence of 2 mM ATP to determine any change in the inhibition of Glo-II activity by this nucleotide.

Chemical Modification Studies with Glo-II-s

Chemical modification of histidine residues by incubation with the histidine-specific reagent, diethylpyrocarbonate (DEPC), was done at 1°C using 1-4 U of Glo-II-s in 30 mM MES, pH 6.0. The modification reaction was started by the addition of an aliquot of a DEPC / EtOH stock solution (1% EtOH, 6 mM DEPC final concentration in the incubation). Aliquots were removed at appropriate time intervals and assays were conducted as previously described without and without 2 mM ATP. A control with no reagent added was treated similarly.

Chemical modification of arginine residues by incubation with phenylglyoxal was done at 1°C using 1-4 U of Glo-II-s in 100 mM TRIS, pH 7.7. The modification reaction was started by the addition an aliquot of phenylglyoxal / DMSO stock solution (5% DMSO, 5 mM phenylglyoxal final concentration). Aliquots were removed and assays were conducted as previously described, without and without 2 mM ATP. A control with no reagent added was treated similarly.

Chemical modification of serine residues by incubation with PMSF was done at 1°C using 1-4 U of Glo-II-s in 100 mM TRIS, pH 7.4. The modification reaction was started by the addition of an aliquot of PMSF/EtOH stock solution (2 mM PMSF final concentration). Aliquots were removed and assays were conducted as previously

described, with and without 2 mM ATP. A control with no reagent added was treated similarly.

Studies on the effect chelation agents, such as EDTA and EGTA, were done at 1°C using 1-4 U of Glo-II-s in buffer A. The incubation was started by the addition an aliquot of a stock solution of chelation reagent (2 mM final concentration). Aliquots were removed and assays were conducted as previously described, with and without 2 mM ATP. A control with no reagent added was treated similarly.

Chemical modification of sulfhydryl residues was carried out at 1°C in buffer A. Concentrated solutions of Glo-II-s (50 U / mL) were incubated under these conditions in the presence of 5 mM DTNB, NEM, GSH, DTA, GSSG, H_2O_2, and Diamide for 200 min.. A control containing no sulfhydryl group reagent was incubated similarly. Aliquots were removed from the incubations at zero time, diluted 1,250 fold, and assayed in the presence and absence of 2 mM ATP. At the end of the 200 minute incubation period, all samples were diluted as above and assayed in the same manner. The final concentration of the sulfhydryl group reagent in the assay after dilution was 4 μM. Reactions were started with the addition of substrate. Results are recorded as the percent of original activity and as the percent inhibition of the post-incubation control.

The effect of particular sulfhydryl - modifying reagents, DTT and DTA, upon Glo-II preparations that exhibited differing extents of inhibition by ATP (due to the loss of sensitivity) were also investigated further. The concentration of stock solutions of the sulfhydryl group reagents were standardized by spectrophotometric determination of the chromophore produced by incubation of the reagent with the sulfhydryl group reagent

DTNB (ε = 13,600 at 412 nm). The residual amount of reagent present during the incubation periods was determined in the same manner. Incubations were conducted in buffer A at 1°C. Samples were removed from the incubation mixtures at appropriate time intervals and diluted 50-fold into buffer A containing 0.2 mM DTNB in order to quench any further reactions between the enzyme and reagent. Controls with no reagent present were treated similarly. The enzyme activity was then assayed in the absence and presence of 2 mM ATP and compared to controls.

Removal of DTA from Glo-II after Incubation with DTA

Removal of the residual DTA from the enzyme after incubation was achieved by adding an excess of DTNB to the solution to quench any further modification reaction. Four volumes of cold acetone (-20°C) were then added slowly to the solution with gentle mixing, and the solution was incubated for 6 hr. at -20°C. The solution was then centrifuged at 14,000 x g for 1 hour at 5°C. The supernatant solution, which contained the DTA-DTNB complex, was discarded. The pellet was then dissolved in 1 mL of ice cold buffer and the process was repeated. The resulting Glo-II sample was then used for further kinetic studies.

Gel Filtration of Glo-II

Concentrated Glo-II (50 uL of a 20 U / mL solution) was applied to a G-75 Sephadex column (50 cm $\times$ 1 cm), that had been equilibrated with 3 volumes of buffer A

containing 100 mM NaCl. Fractions (0.25 mL) were eluted from the column at a flow rate of 0.25 mL / minute and assayed as previously described.

The column was then equilibrated as before with the same buffer medium containing 1 mM ATP. Again, concentrated Glo-II (50 uL of a 20 U / mL) was applied to the column, eluted with the modified buffer solution, and assayed as before.

Fast Performance Liquid Chromatography (FPLC) of Glo-II Samples

The elution profiles of Glo-II samples were determined on a Superdex 200 HR 10/30 high performance gel filtration column. The column was first equilibrated with a buffer medium containing 40 mM TES and 100 mM NaCl buffer at pH 7.2. Samples containing 50 ug of purified Glo-II, 100 uL volume, in buffer A, pre-filtered with a 0.45 μm filter were then applied to the column. Protein was eluted from the column at a flow rate of 0.5 mL / min. Fractions (0.25 mL / fraction) were collected and as assayed as described previously.

Circular Dichroic (CD) Analysis of Glo-II

Purified samples of Glo-II were analyzed by circular dichroic analysis over the far-ultraviolet CD spectra, range 190 – 260 nm, 0.1 cm path length, constant bandwidth of 1.50 nm, at 15°C with a Jasco spectropolarimeter. Spectra were analyzed using 3 seconds of integration time per reading, and were repeated 3 times. Signals at each wavelength were then averaged and digitally filtered by an AVIV 60DS version 4.1g digital-smoothing program. The process was repeated using appropriate blanks and

spectra were corrected accordingly. The concentration of protein in the sample was 8 μM. The buffer used was 10 mM TRIS, pH 7.4.

Temperature Denaturation of Glo-II

Purified samples of Glo-II were denatured and analyzed by circular dichroic analysis at 220 nm, 0.1 cm path length, constant bandwidth of 1.50 nm, with a Jasco spectropolarimeter. Readings were analyzed using 3 seconds of integration time per reading, and were repeated 3 times. The range of temperatures in which data were collected was from 25°C to 89°C, with temperature steps of 2.0°C, 0.2 min. equilibration time. Signals at each temperature were then averaged and digitally filtered by an AVIV 60DS version 4.1g digital-smoothing program. The process was repeated using appropriate blanks and spectra were corrected accordingly. The concentration of protein in the sample was 8 μM. The buffer used was 10 mM TRIS, pH 7.4.

Tryptophan Fluorescent Emission Spectra of Glo-II Samples

The tryptophan fluorescent emission spectra of purified samples of Glo-II were analyzed in an SLM-Amico Bowman II luminescence spectrometer, excitation wavelength 280 nm, over a range of 310 to 500 nm. Further analysis of the samples was conducted by anisotropic analysis of the spectrum over the same range of wavelengths. The protein concentration of all samples analyzed was 8 μM. Buffer used was 10 mM TRIS, pH 7.4.

Sulfhydryl Group Determination of Glo-II

Sulfhydryl determination of purified Glo-II-s and Glo-II-i preparations were done according to the method of Kosower (236). Typically, 20 uL of a 50 mM solution of monobromobimane was added to 200 uL of an 8 μM solution of Glo-II preparations in 10 mM TRIS buffer, pH 7.4. Glo-II preparations, 8 μM in 10 mM TRIS, 200 uL, were also treated with 6 M urea before the addition of the bimane solution. The fluorescent emission spectra of purified samples of Glo-II were analyzed in an SLM-Amico Bowman II luminescence spectrometer over a range of 400 to 600 nm as previously described (237). Appropriate controls, with no enzyme added, were treated similarly for comparison to enzyme solutions.

CHAPTER III

RESULTS

Glo-II-s - Purification

The purification of Glo-II-s from bovine calf liver was successfully accomplished by the rapid affinity chromatography method described in the Methods section. Table VIII summarizes the data from a typical purification of Glo-II-s. The 612-fold purified enzyme exhibited a specific activity of 1549 U / mg of protein. The above purification factor is based on the 14,000 × g supernatant fraction obtained from a calf liver homogenate.

Glo-II-s – Estimation of Molecular Weight and Analysis of Purity.

Molecular weight estimation and analysis of the purity of the purified bovine calf liver Glo-II-s were obtained by SDS-PAGE (Figure 1). Both Coomassie blue (not shown) and silver staining (Figure 1) were used to analyze the purity of the samples. Coomassie blue staining procedures showed that Glo-II-s appears to be a single polypeptide chain that has been purified to homogeneity. Silver staining procedures, at times, revealed minor impurities in the samples, although the purity of the samples was estimated to be as high as 95 percent pure. The relative mobility of the protein sample after purification (lane 2) was compared to that of a set of standard molecular weight proteins (lane 1) and analysis of this comparison gave an approximate molecular mass of 29,000 daltons.

Glo-II-s - Stability with respect to temperature.

We had observed in our initial experiments with the purified fractions of Glo-II-s that the enzyme activity appeared unstable at elevated temperatures. Therefore, the stability of Glo-II-s at a variety of temperatures was determined in order to set temperature parameters to be used in future studies.

Long term storage of concentrated aliquots of Glo-II-s at -80°C was typically limited to approximately two months of storage before a significant loss of activity (>10%) was detected. Upon removal from these storage conditions and dilution, enzyme activity proved to be relatively stable for at least 1 hr. when incubated at 12°C or below but rapidly lost activity when stored at higher temperatures (Figure 2). At 17°C, more than 30 percent of the initial activity was lost over a 1 hr. period. At 22°C, over 50 percent of the initial activity was lost in the same time period.

Since the long-term stability of Glo-II-s was limited, a number of precautions were taken. Purified samples of concentrated Glo-II-s were divided into small aliquots and stored at -80°C until use. Typically, aliquots were removed from the -80°C storage conditions, diluted with ice cold buffer, and assayed under standard conditions to determine if any significant loss of activity had occurred during storage. All experiments were conducted on samples that exhibited only minor losses of activity during storage (<10%).

Since the short-term stability of Glo-II-s during experiments was also limited, additional precautions were taken. During experiments, the diluted aliquots of enzyme was maintained on ice until use and assayed periodically to detect any losses of activity.

Incubation experiments were also conducted at or below 12°C whenever possible. Control samples of enzyme were included in all experiments to determine if any changes in enzyme activity were due to the instability of the enzyme. Assays, which were typically done at 25°C, were conducted over 45 sec. periods to minimize any loss of activity.

Glo-II-s – Stability with respect to pH.

In order to set the experimental parameters to be used in subsequent studies, the stability of Glo-II-s with respect to pH was determined. Studies were conducted to determine the stability of Glo-II-s during short-term incubation of the enzyme over a wide range of pH values. Aliquots of Glo-II-s were incubated at pH values from 4 to 10 for 5 min. at 1°C and assayed under standard conditions (see Methods). Experiments revealed that Glo-II-s activity is stable under these conditions over a pH range from 5.5 to 8.5 (Figure 3). A rapid loss of enzyme activity was detected when samples were incubated at pH conditions outside of this range. Due to these results, all further experiments were conducted within a pH range of 5.5 to 8.5.

Glo-II-s – Activity with respect to pH.

Studies were conducted to determine the activity of Glo-II-s over the range of pH values from 5.5 to 8.5. The determination of Glo-II activity is dependent upon the spectrophotometric detection of the GSH-DTNB complex, but the extinction coefficient of this complex changes dramatically over the pH range used in these studies. Furthermore, the rate of the formation of the GSH / DTNB chromophore may also

change over the pH range used in these experiments. Experimental conditions were modified such that the effect of aforementioned changes in the characteristics of the GSH – DTNB complex due to pH would not effect the results.

Experimental conditions were set as follows. First, reaction mixtures at the specified pH were incubated, without DTNB present, for a predetermined time period over which the reaction was shown to be linear. Second, reactions were quenched by the addition of acid. Finally, the pH was adjusted to pH 7.3 by the addition of concentrated buffer / DTNB solutions and incubated at 22°C for five min. before readings were taken (see Methods section). Control mixtures with substrate present but no enzyme added were treated similarly. No change in the stability and / or spontaneous breakdown of the substrate was observed under these conditions; also, the rate of chromophore formation and the extinction coefficient for that chromophore were consistent in all samples.

A graphical representation of the activity versus pH profile of Glo-II-s is shown in Figure 4. Glo-II-s activity exhibited a steady increase with an increase in pH over the pH range tested.

Glo-II-s – Inhibition of activity by nucleotides with respect to pH.

In order to determine the effect of pH on the inhibition of Glo-II-s by nucleotides, the experiments described above were repeated in the presence of nucleotide and treated in the same manner. A graphical representation of the Glo-II-s activity in the presence of nucleotide versus pH profile is also shown in Figure 4. The raw data from this graph were utilized to construct Figure 5, which depicts the percent inhibition of Glo-II-s activity in the presence of nucleotide versus increasing pH. It is clear from these data

that significant inhibition of enzyme activity by nucleotides occurs over a narrow pH range, 6.5-8.0, and that inhibition is greatest at physiological pH (7.4). It is as yet unclear whether the results are due to the titration of a residue in the protein or the phosphate residue of the nucleotide.

Inhibition Studies with Nucleotides.

Since both ATP and GTP were found to inhibit Glo-II-s activity, the specificity of the inhibition of Glo-II-s by nucleotides was in question. In order to determine if the inhibition was restricted to any one class of nucleotide; or if certain nucleotides were significantly more inhibitory than others, Glo-II-s was assayed in the presence of wide variety of nucleotides. Assays were at fixed substrate concentrations (0.25 mM SLG) in the presence of a fixed concentration of nucleotide (2 mM) and the activities were compared to that of a control (no nucleotide).

Figure 6 shows a typical representation of the results of these experiments. Other nucleotides (CTP, NAD^+, NADH, $NADP^+$, NADPH, FMN, pppGpp, ppGpp, ApppA, ADPR, and PolyA) were tested as well, but since they showed no, or very little, inhibition of Glo-II-s activity, they are not shown in the figure. Among the nucleotides tested, we found that inhibition of the Glo-II-s activity is essentially restricted to four compounds: ATP, ADP, and GTP, GDP. It is apparent that only purine nucleotides possessing a phosphoanhydride bond are effective inhibitors, with ATP appearing only slightly more inhibitory than the other three.

Incubation Studies with Nucleotides.

Although ATP appeared slightly more inhibitory than the other purine nucleotides, the enzyme exhibited no clear preference to any of the four inhibitory nucleoside di- and tri-phosphates. We also investigated the effect of pre-incubation of the enzyme with various nucleotides (1.4 mM), followed by dilution of the incubated samples into the assay mixture giving a non-inhibitory concentration of nucleotide. The results provided further insight into the specificity / effects of nucleotide interaction with Glo-II-s. Figure 7 summarizes the results.

Of the nucleotides tested in this manner, only ATP had any significant effect on the enzyme. Even after dilution of the Glo-II-s / ATP samples to non-inhibitory levels of ATP, a significant decrease in both activity and the inhibition of Glo-II-s activity, when assayed in the presence of ATP, was observed. Attempts to recover the activity or the original levels of nucleotide inhibition after dilution of the enzyme were met with failure. Although incubation of the enzyme with GTP does cause a moderate decrease in the Glo-II-s activity (-10%), no effect on the inhibition of Glo-II-s by ATP was observed. Thus, ATP was used as the model inhibitor for further experiments.

Glo-II-s - Kinetic parameters using ATP as a model inhibitor.

Figure 8 depicts the percent inhibition of Glo-II-s activity by ATP at fixed SLG concentration (0.25 mM) and increasing ATP concentrations (0-10 mM). The data show that 100% inhibition of Glo-II-s activity by ATP could not be achieved, even at levels of inhibitor as high as 10 mM. Similar results were obtained with GTP. Values for maximum inhibition typically ranged from 60-70%. In pure inhibitor systems, infinitely

high concentrations of inhibitor will result in the velocity of the enzyme eventually reaching zero, as the EI complex is incapable of producing product (238). In the case of a partial inhibitory process, infinitely high concentrations of inhibitor will result in a modified form of the enzyme (EI) which is still able to bind to the substrate (ESI) and produce product but at a reduced rate (238). Therefore, graphical analysis of a percent inhibition versus I plot, in the case of partial inhibition, will result in a hyperbolic curve and plateau at the level of maximum inhibition (the point where all enzyme is in the EI form). The hyperbolic nature of the curve resulting from these data suggests ATP or GTP act as a partial inhibitor of Glo-II-s activity.

Figure 9 is a Lineweaver-Burk plot for the inhibition of purified Glo-II-s by ATP. Inhibition appears purely non-competitive. The K_M for the natural substrate (0.54 mM SLG) is approximately 2.5 – 3.5 fold higher than previously reported values (0.18 – 0.22 mM SLG). Similar results were obtained using GTP.

Of interest was the information obtained from the replots of the experimental data. Figure 10 shows the effect of ATP on Glo-II-s activity as analyzed by a Dixon replot. Figure 11 shows the effect of ATP on Glo-II-s activity as analyzed by a slope versus inhibitor concentration replot. Both replots are obviously parabolic.

Taken together, the data suggests parabolic-parabolic partial non-competitive inhibition of Glo-II-s activity by ATP (or GTP) and supports the presence of an ESI complex that is still capable of producing product but at a reduced velocity. Furthermore, the finding of partial noncompetitive inhibition and parabolic replots is known to result from the combination of the inhibitor with two enzyme forms (238). The presence of two forms of the enzyme was further supported by the non-linearity of the Eadie-Schatchard

plot of kinetic data using the same enzyme (Figure 12). Therefore, the data suggest the presence of two forms of the enzyme in samples tested.

Glo-II-s - Investigation of conditions which cause the loss of inhibition by ATP.

As stated earlier, we had observed the loss of Glo-II-s activity over time even under optimal storage conditions (- 80°C). We had also observed a concurrent loss of the inhibition of Glo-II-s activity by ATP under the same conditions. This observation was explored in further detail.

Figure 13 summarizes the results of a typical incubation of Glo-II-s samples when incubated at 12^{o}C over time. Aliquots of the enzyme solution were removed from the incubation periodically and assayed in the absence of ATP (uninhibited activity) and in the presence of ATP (inhibited activity). The uninhibited activity of the enzyme undergoes a steady decrease in activity with time (in this particular experiment the time period was 33 hr.). At the end of this period, the remaining activity of the enzyme is unaffected by the presence of ATP. No further changes in either the uninhibited or inhibited activity of the enzyme occurred over the next 24 hr. (data not shown).

As stated earlier, previous experiments involving the short-term incubation of Glo-II-s with ATP showed a decrease in both the uninhibited activity of the enzyme and the inhibition of enzyme activity by ATP (Figure 7). Figure 14 shows the typical effect of incubation of the enzyme in the presence of high levels of ATP (5mM) under "stable" incubation conditions (concentrated Glo-II-s at 1^{0}C for 2 hr.). After incubation, samples were diluted to non-inhibitory levels of ATP (6 μM) and assayed for both activity and inhibition by ATP. As in the temperature desensitized sample of Glo-II-s, a loss of both

enzyme activity as well as the inhibition of activity by ATP had occurred. Attempts to recover either the activity or the inhibitory properties of ATP after dilution met with failure in earlier experiments.

Both procedures that cause the loss of sensitivity of Glo-II-s preparations presented here have proved to be consistent methods for producing Glo-II-i samples. Furthermore, the samples can be re-purified by the affinity chromatography step of the purification process presented earlier.

Glo-II-i - Kinetic parameters.

Glo-II-i, obtained by the desensitization of Glo-II-s by incubation at 12^{o}C, was re-purified using the last two steps of the purification process (acetone precipitation and affinity chromatography) to remove any denatured protein from the samples. The resulting samples were then used in the following experiments.

Figure 15 compares the percent inhibition of Glo-II-s activity by ATP to that of the Glo-II-i samples desensitized by incubation at 12°C. Both samples were assayed at fixed SLG concentration (0.25 mM) with increasing ATP concentrations (0-10 mM). The ability of ATP to inhibit Glo-II-i is still apparent, although the maximum levels of inhibition (< 20%) at very high levels of ATP (10 mM) are greatly reduced when compared to those of the Glo-II-s samples assayed under the same conditions.

Figure 16 shows the Lineweaver-Burk plot for the purified Glo-II-i sample after temperature desensitization and re-purification. The enzyme exhibits linear kinetics over the substrate range used and the value for the K_M is 0.22 mM for the substrate, SLG. The specific activity of the enzyme was 750 U / min. / mg. These values are in close

agreement with previously reported values (Table III). In Figure 17, an Eadie-Scatchard replot of the data from Figure 16 shows linearity. Such linearity is found in such replots when only one kinetically distinguishable form of an enzyme is predominant in the sample (238).

Glo-II-i, after desensitization by incubation with ATP, was also re-purified using the last two steps of the purification process to remove any denatured protein and residual ATP from the sample. Figure 18 compares the percent inhibition of Glo-II-s activity by ATP to that of the Glo-II-i samples desensitized by incubation with ATP. Both samples were assayed at fixed SLG concentration (0.25 mM) with increasing ATP concentrations (0-10 mM). As with the Glo-II-i preparation desensitized by incubation at 12°C, nucleotide inhibition of the ATP-desensitized Glo-II-i also remains apparent but is very much greatly reduced.

Figure 19 shows the Lineweaver-Burk plot for the re-purified Glo-II-i sample desensitized by ATP incubation and then re-purified. Lineweaver-Burk analysis gave a linear plot with a K_M = 0.22 mM SLG. The specific activity of the enzyme was 650 U / min./ mg. Again, these values are in close agreement with previously reported values (see Table III). The values determined for the Glo-II-i samples desensitized by incubation at 12°C and by incubation with ATP are also in close agreement. In Figure 20, an Eadie-Scatchard replot of the data from Figure 19 again gives a linear plot, suggesting that only one kinetically distinguishable form of the enzyme is present after this process.

The two procedures described above result in what appear as kinetically similar enzymes. Both procedures result in a preparation of Glo-II-i that exhibits only one

detectable form of the enzyme as determined by the Eadie-Schatchard replots and have similar K_M values for SLG. Finally, both procedures result in a form of Glo-II that is no longer significantly inhibited by ATP.

Each of these "desensitization" procedures can be separated into two steps; incubation and re-purification. Analysis of the kinetic characteristics of the enzyme after each individual step was then completed in order to determine at which point in the procedure that the K_M value of the enzyme for the substrate had changed.

With respect to the Glo-II-i sample formed by incubation of Glo-II-s at 12°C, the enzyme activity gave similar K_M values (0.22 mM) either at the end of the incubation period or after re-purification (data not shown). As stated earlier, the Glo-II-s enzyme before the incubation period gave a K_M value of 0.54 mM . Thus, the change in K_M value for the Glo-II-i sample formed by incubation at 12°C occurs during the incubation period.

The Glo-II-s enzyme was also incubated with ATP (5 mM) as previously described. At the end of the incubation period, the enzyme was diluted to a non-inhibitory concentration of ATP (6 μM). The enzyme activity at the end of the incubation period resulted in a K_M value of 0.53 mM, a value in close agreement with the K_M value of 0.54 mM for the Glo-II-s sample before incubation (data not shown). Thus, the change in K_M value for the Glo-II-i sample desensitized by incubation with ATP does not occur during the incubation period.

The Glo-II-s enzyme was also incubated with ATP (5 mM) and re-purified as previously described. After the removal of the ATP by re-purification, the resulting Glo-II-i sample was compared by Lineweaver-Burk analysis at equal protein concentrations to the original Glo-II-s (before incubation with ATP and re-purification). Figure 21

summarizes the results. The enzyme after re-purification exhibited the characteristic reduction in K_M value (from 0.54 mM to 0.22 mM) that had been described previously. No significant change in the Vmax was observed. The initial velocity of the Glo-II-i enzyme was consistently greater than that of the Glo-II-s enzyme at the substrate concentrations tested.

Glo-II-s vs.Glo-II-i - Investigation of the Structural and / or Conformational Differences.

Studies were conducted in order to compare possible structural differences between Glo-II-s and Glo-II-i preparations. It was postulated that the different kinetic characteristics between the Glo-II-s and Glo-II-i preparations are due to changes in enzyme conformation.

Figure 1 compares the molecular weight of the of the Glo-II-s sample (lane 2) to that of either Glo-II-i samples discussed above (lanes 3 & 4) by SDS-PAGE, detected by silver staining. It is apparent that the molecular weight of the enzyme, as well as the relative mobility of the protein under these conditions, remains unchanged. Therefore, it is assumed that the loss of sensitivity to nucleotide inhibition is not due to any change in molecular weight that could be detected under these conditions.

Figure 22 shows a comparison of the elution profiles of the activity of Glo-II-s and Glo-II-I, desensitized by incubation at 12°C, samples as determined on a Superdex 200 HR 10/30 high performance gel filtration column. The graph clearly shows that the two samples do not significantly differ in their elution patterns. Gel filtration (using a G-75 Sephadex gel filtration column) of the Glo-II-i sample desensitized by incubation with ATP gave similar results (data not shown). Therefore, it is assumed that the loss of

sensitivity to nucleotide inhibition was not due to any large change in molecular shape or size that could be detected by these methods.

Further experiments using the G-75 Sephadex gel filtration elution pattern of Glo-II samples under varying conditions were also performed. Equilibration of the filtration column with 1 mM ATP did not change the elution profile of Glo-II-s activity when compared to the elution profile of the same protein under the same conditions without ATP present (Figure 23). Similar results were obtained for the Glo-II-i samples as well (data not shown). Therefore, the addition of ATP does not cause any large change in the molecular shape of the enzyme samples under these conditions.

Figure 24 shows a comparison of the circular dichroic spectrum of the Glo-II-s sample as well as the Glo-II-i sample desensitized by incubation at 12°C. The circular dichroic spectrum of Glo-II had been previously reported, identifying the protein as a typical alpha / beta protein (183). No significant difference between the CD spectrum of the Glo-II-s and the Glo-II-i samples was apparent. The spectrum produced for either Glo-II sample was also in close agreement with that of previously published data. Therefore, the loss of sensitivity to nucleotide inhibition is not likely due to the loss of, or large changes in, the native structure of the protein.

Figure 25 shows a comparison of the heat denaturation temperatures of Glo-II-s and Glo-II-i samples as analyzed by the circular dichroism of the proteins at 220 nm. Both proteins show similar temperature denaturation curves in which total loss of secondary structure does not occur until temperatures approaching 80°C. Therefore, the loss of sensitivity to nucleotide inhibition is not likely to be due to unfolding of the native structure of the protein.

Since no gross structural changes in the two proteins could be detected by any of the tools employed, it was considered possible that the loss of Glo-II sensitivity is due to micro-environmental changes in the conformation of the protein. Since the fluorescence emission spectra of the tryptophan residues of proteins is often used to detect such small changes in protein conformation, a comparison of the emission spectra of equal molar amounts of the Glo-II-s sample and the Glo-II-i sample desensitized by incubation at 12°C was made. Figure 26 shows the results.

Although the amino acid sequence of calf liver Glo-II has not been determined, the sequence of human liver Glo-II has been reported. The amino acid sequence of the enzyme from human liver shows that only two tryptophan residues are present in the protein (202). As stated earlier, available sequence data show strong sequence homologies from species to species and that the enzyme likely evolved from a common ancestor (203). Therefore, the presence of two tryptophan residues can be assumed to exist in the sequence of calf liver Glo-II.

Figure 26 compares the tryptophan fluorescent emission spectra of the two Glo-II samples tested. The differences in the spectra are clear in that the Glo-II-i sample exhibits a large degree of quenching of the emission signal at the lower wavelengths analyzed when compared to that of the Glo-II-s sample. The quenching of the signal is assumed to be due to the greater exposure of this residue to the outer, aqueous environment of the protein. The obvious differences in the microenvironment of the tryptophan residues of the two forms of the enzyme suggests that the protein conformation has undergone a small change during the transition from the nucleotide sensitive to the nucleotide insensitive form.

Excitation of fluorescent residues by plane polarized light (anisotropy) furnishes a method of further resolution of the heterogeneous fluorescence in proteins that possess multiple fluorescent residues (239). If an excited residue is able to move or rotate appreciably before the fluorescent light is emitted, the fluorescence will be depolarized to some extent and therefore the signal will decay at a faster rate than a residue that is not able to move as freely. Therefore, a conformational change in a protein is often detectable by changes in the ability of the residues to move or rotate, as determined by anisotropy.

The assumption that a conformational change occurs in the transition of Glo-II-s to Glo-II-i, suggested by the comparison of the fluorescent emission spectra of the two forms of the protein, was further supported by comparison of the anisotropic derived fluorescent spectra of the two proteins. Figure 27 compares the fluorescent emission spectra of the two protein forms under these conditions. As in the previous experiment, the differences in the two spectra support that a conformational change in the protein has occurred in the transition of Glo-II-s to Glo-II-i.

Glo-II-s –Chemical Modification Studies

A series of chemical modification studies were conducted on samples of Glo-II-s in order to investigate the effect of such modifications on both the activity and inhibition of Glo-II by nucleotides. Results from this study were compared to results from chemical modification studies on Glo-II preparations previously reported in literature.

Previous chemical modification of amino acid residues on purified Glo-II indicated both a critical histidine and an arginine in the active site (182, 184). Chemical

modification of the histidine residues of Glo-II-s by diethylpyrocarbonate (Figure 28), and of arginine residues by phenylglyoxal (Figure 29)decreased activity similarly to that previously reported, but had no effect on the inhibition of Glo-II by ATP. Earlier studies involving chemical modification of serine residues by PMSF had no significant effect on the activity of the enzyme (182, 184). Chemical modification of serine residues by PMSF had no effect on the activity or nucleotide sensitivity of Glo-II-s (data not shown). The data suggest that the inhibition of Glo-II-s activity by ATP does not involve the arginine, serine, or histidine residues of Glo-II.

Studies on the effect of the addition of divalent ions or chelation agents were also conducted due to reports of the binding of a zinc ion to Glo-II and its proposed requirement for catalytic activity (191). The addition of EDTA, EGTA, or 8-hydroxyquinoline to incubations of pure Glo-II-s exhibited no effect on either enzyme activity or its inhibition by ATP (data not shown). Furthermore, the addition of divalent metal ions (Zn^{2+}, Mn^{2+}, or Ca^{2+}) to the enzyme produced no effect on either enzyme activity or its inhibition by ATP (data not shown). Of notable interest was the lack of any requirement for Mg^{2+} with respect to the inhibition of enzyme activity by ATP (data not shown).

Glo-II – Effect of Sulfhydryl Group Modification on Activity and Inhibition by ATP

Uotila reported the stabilization of purified Glo-II from human liver by the addition of high concentrations of reducing agents such as BME and DTT (168). Other studies have reported the loss of Glo-II activity upon the addition of sulfhydryl group reagents, including alkylating agents (iodoacetate), oxidizing agents ($HgCl_2$), and agents

known to react with disulfides ($CdCl_2$ and Arsenite) (168, 182, 184). Since no protection from the loss of activity was observed in the presence of slow substrate, the presence of free thiol group(s) located away from the active site, but somehow involved in the hydrolase activity of Glo-II, was proposed (184).

The deduced amino acid sequence of human liver Glo-II reveals a 260 amino acid protein with six cysteine residues found dispersed between residues 104 to 171 (202). As stated earlier, Glo-II is predicted to be organized into two distinct domains (183). The N-terminal domain, residues 1-180, is predicted to be a compact, stable domain consisting exclusively of beta-sheet secondary structure in which the active site is present. Therefore, all of the cysteine residues are predictably found in the N-terminal domain. To date, no analysis of the number of free sulfhydryl groups present in Glo-II has been reported.

As stated earlier, purified Glo-II-s activity proved to be relatively unstable. Attempts to stabilize the activity by the addition of DTT proved futile (data not shown). Notably, these results were contrary to those previously reported by Uotila (168). Experiments were also conducted using mono-bromobimane, a fluorescent reagent highly specific for free sulfhydryl groups, to determine if any free cysteine sulfhydryl groups were present in the protein. Neither Glo-II-s nor Glo-II-I, desensitized either by incubation at 12°C or by incubation with ATP, exhibited the presence of free cysteine groups under both native or denatured by the addition of 6 M urea states. Therefore, we assumed that the cysteine residues of bovine calf liver Glo-II are possibly in the oxidized form, likely as three disulfide groups.

These findings were further supported by studies of the effect of sulfhydryl reagents on Glo-II-s activity or inhibition of its activity by ATP. Results are summarized in Figure 30. Neither NEM, GSH, GSSG, or DTNB exhibited any effect on Glo-II-s activity or its inhibition by ATP. Diamide, a reagent known to cause the formation of disulfide bonds with accessible free sulfhydryl residues (240), did exhibit a minor effect on enzyme activity, but no significant effect on the sensitivity of the enzyme to ATP was observed. Longer incubation periods of the enzyme in the presence of these reagents produced no further effect (data not shown).

Of great interest was the effect of chemical modification of Glo-II-s with Meos-2,5-dimercapto-N,N,N',N'-tetramethyl-adipamide (DTA), a reagent known to efficiently cleave disulfide bonds at physiological pH. Incubation of purified Glo-II-s in the presence of a low concentration of DTA (1 mM) produced a moderate decrease in the activity of Glo-II-s. Surprisingly, the reagent also produced a moderate increase in the inhibition of the sample by ATP (from 42-50 %). Although the increase in inhibition was slight, it warranted further study.

Figure 31 depicts a typical time course study of the effect of DTA on the activity of the Glo-II-i enzyme desensitized by incubation at 12°C. Incubation was done in the presence of a high concentration of the reagent (4 mM) at 1^0C over 10 hr.. Results show an initial period of stability (5 hr. in this case), followed by a period of significant loss of enzyme activity. Figure 32 shows the effect of DTA on the same enzyme solution with respect to inhibition by ATP. Results show that during the initial period of stability, inhibition of the enzyme activity by ATP (2mM) increased steadily. No significant change in inhibition was observed after the enzyme had begun to lose activity. Similar

results were found with the Glo-II-i desensitized by the addition of ATP (data not shown).

Experiments were also performed on the Glo-II-s form of the enzyme under the same conditions (4 mM DTA, 1^0C). In contrast to the initial period of stability observed during the incubation of the Glo-II-i samples with DTA, the Glo-II-s form of the enzyme exhibited immediate and significant losses of activity in the presence of the reagent (Figure 33). In contrast to the immediate increases in the level of inhibition of enzyme activity by ATP observed during the incubation of the Glo-II-i samples with DTA, the Glo-II-s form exhibited only moderate increases in the ability of the enzyme to be inhibited by ATP (Figure 34).

The differences in the effects of DTA on the two forms of the enzymes led us to investigate the kinetic parameters of the enzyme forms after modification. The DTA was removed from these samples and used in kinetic studies. Figure 35 shows the Lineweaver-Burk plot for the inhibition of DTA-modified Glo-II-s sample by ATP. It was determined that ATP still binds non-competitively to DTA-modified Glo-II. Furthermore, the K_M for the natural substrate (0.6 mM SLG) is significantly higher than previously reported values (0.18 – 0.22 mM SLG), and slightly higher than the K_M for the Glo-II-s fraction reported earlier herein (0.54 mM SLG). Figure 36 shows the effect of ATP on the DTA-modified Glo-II activity as analyzed by a Dixon replot. Figure 37 shows the effect of ATP on DTA modified Glo-II as analyzed by a slope versus inhibitor concentration replot. Replots show that the parabolic curve of the original mixture of enzyme forms in the Glo-II-s preparation is no longer evident. Figure 38 shows a linear Eadie-Schatchard plot of kinetic data obtained from the same enzyme sample. Attempts

to reach the level of 100% inhibition met with failure, with the maximum level of inhibition typically reaching no more than 70% (data not shown). Similar results were obtained from the Glo-II-i samples (data not shown).

CHAPTER IV

DISCUSSION

Glo-II-s - Purification

With but one exception (25), there is no mention of purine nucleotide inhibition of Glo-II, in crude or purified preparations, in the literature. Little, if any, purine nucleotide inhibition of Glo-II is found when the enzyme is purified by traditional methods, which require multiple chromatography steps and may take days to complete. The process for the purification of Glo-II developed in this laboratory is a very rapid method (12 hours or less) that requires only a single chromatography step. The resultant enzyme, Glo-II-s, is significantly inhibited by physiological concentrations of ATP or GTP.

Attempts to define any single step or reagent in previously used purification methods that was responsible for the loss of inhibition of Glo-II-s activity by ATP met with failure (Norton, S., Giovanni, B., and Elia, C., unpublished results). In earlier studies, attempts to purify large quantities of the enzyme in this "sensitive " form using the same basic protocol presented here, but requiring a longer period of time, generally resulted in a significantly decreased sensitivity to purine nucleotide inhibition (Norton S. and Elia, C. unpublished results). It was not until we adopted a more rapid method of purifying small quantities of the enzyme that the purification process resulted in an enzyme that was "sensitive" to purine nucleotide inhibition. Such observations suggest

that the length of time and / or the complexity of the method used to purify the enzyme may affect an important quality of Glo-II, structure or conformation, which confers sensitivity to certain purine nucleotides.

We have found that purified Glo-II-s preparations are relatively unstable. Even at - 80°C, the enzyme activity and / or sensitivity undergoes significant losses over time. At higher temperatures, the loss of these characteristics are observed to be quite rapid (Figure 13). In contrast, the Glo-II activity and / or sensitivity of both the crude homogenates and the acetone fractions have proved to be relatively heat-stable (data not shown). Thus, it is likely that the instability of the enzyme begins at, and is a consequence of, the removal of the protein from stabilizing components of the cytosolic milieu during the purification steps. A rapid and simple method is thus necessary to obtain a purified enzyme that exhibits the same characteristics in the purified state as it does in the crude fractions.

The purification of Glo-II-s by the method described here results in a relatively pure fraction, as analyzed by SDS-PAGE (Figure 1), of highly concentrated Glo-II-s (> 3500 U / ml). The overall yield of the initial enzyme activity was consistently around 40%. The specific activity of the enzyme (> 1500 U / mg) was consistently higher than values previously reported (600-900 U / mg). Finally, the rapid and easy method produces milligram quantities of the purified enzyme in relatively short time periods.

Glo-II-s – Experimental Parameters; pH and Temperature

As stated earlier, the stability of both the activity and the sensitivity of Glo-II-s with respect to temperature is limited. Precautions were taken throughout our experiments to insure our results did not reflect the loss of activity or inhibition qualities of the enzyme. These precautions are described previously and will not be further elaborated upon here.

The effect of pH on the activity of Glo-II has been previously reported (168, 182). Glo-II, purified from the liver of either rat or human, was reported to exhibit a pH optimum of activity from 6.8 to 7.5 and to decrease rapidly outside this range (168, 182). Glo-II-s, purified in the manner presented here, proved to be relatively stable over a broad pH range, pH = 5.5-8.5 (Figure 3), and in contrast to previously reported results, exhibits a steady increase in activity with increasing pH over this range (Figure 4). The differences in the results may be, in part, due to the methodology used. Previous reports did not apparently allow for the possible assay problems related to either the changes in the extinction coefficient or changes in the rate of formation of the GSH – DTNB complex over the pH range tested. Methods used here were designed to account for such potential problems. Significant inhibition of Glo-II-s activity by nucleotides is limited to a very narrow pH range, pH = 6.8-7.6, and is at its greatest at physiological pH (Figure 5). These occurrences are likely due to either the titration of a residue within the protein itself, or the titration of a residue in the substrate or inhibitor.

Glo-II-s – Inhibition Studies

Studies to determine the specificity of the inhibition of Glo-II-s by nucleotides showed that the inhibition of Glo-II-s activity is restricted to four of those compounds tested: ATP, GTP, ADP, and GDP (Figure 6). Pre-incubation experiments with the enzyme and each of of these four nucleotides gave further insight into the inhibitory differences of these nucleotides (Figure 7). Only ATP was found to cause any significant change in the enzyme activity or the inhibition of the enzyme activity by nucleotides, following dilution. Since other nucleotides could not produce this effect when studied under the same conditions, ATP appeared as the most interesting inhibitor / effector of Glo-II-s and was therefore used in subsequent experiments to determine the nature and significance of its effects.

Glo-II-s – Kinetics Studies

Kinetics studies on the purified Glo-II-s enzyme showed linear plots over the substrate range tested when analyzed by the double reciprocal plot method of Lineweaver-Burk; the Km value for SLG was determined to be 0.54 mM (Figure 9). This value is substantially higher than that reported for the human liver (0.146 mM) (168). Although the magnitude of the differences in Km values could possibly be attributed to differences in procedure / methodology, the presence of a form of Glo-II not yet studied, with a higher Km value for SLG may be an alternative explanation.

The data suggest that the inhibition of Glo-II-s activity by ATP is partial non-competitive in nature (Figures 8, 9). Thus, the inhibitor forms a complex with the

enzyme (EI) that is still capable of binding to the substrate (ESI) but the ESI complex produces product at a reduced rate (238). The enzyme purified by the method presented here not only exhibits a higher Km value for the substrate; the maximum velocity of the enzyme, in the presence of inhibitory concentrations of ATP, is also greatly reduced.

Replots of the kinetic data, employing both slope versus inhibitor concentration or Dixon replots, show parabolic characteristics (Figures 10, 11). Parabolic replots occur with partial non-competitive inhibition of enzyme activity only when the inhibitor is binding with two different forms of the enzyme, each form with significantly different affinities for the inhibitor (238). This suggestion was further supported by the non-linearity of the Eadie-Schatchard replot of kinetic data from subsequent experiments with Glo-II-s (Figure 12). These results suggest that the purification of Glo-II by the method presented here results in the purification of two forms of the enzyme, one of that exhibits a significantly higher affinity for ATP than does the other.

The possibility of two forms of the enzyme in the purified fraction was not unexpected. We had already noted that the enzyme lost sensitivity during more conventional purification processes. Our previous studies had suggested that the process involved in the loss of nucleotide sensitivity by purified Glo-II probably began during the chromatography stage of purification. It was not surprising that our process resulted in some percentage of the enzyme reverting to this insensitive form, regardless of how rapid the purification process was. Due to these results, a major focus of our further research was to find methods of producing fractions that contained predominantly only one form of the enzyme.

Glo-II-s – Possibility of a Variant Regulatory Nucleotide Binding Site

The binding of mononucleotides to various proteins, in which a phosphate or adenylate transfer occurs, requires the presence three highly conserved motifs (*Kinase-1, Kinase-2, and Kinase3*) that form a "catalytic" nucleotide-binding site (242). *Kinase-1* is identified by the highly conserved "Walker" sequence, GXXXXGXK. *Kinase-2* contains an invariant aspartate residue that is responsible for the coordination of the required ATP-Mg^{2+} complex during phosphate transfer in these catalytic reactions. Both of these motifs outline the peptide segments that bind the phosphate residues of the bound nucleotide. *Kinase-3* is thought to be the peptide segment that binds to the purine or ribose portion of the nucleotide, thus determining the specificity of the protein-nucleotide interaction. Generally, these proteins are often highly specific for one nucleotide, and rarely are known to bind other nucleotides to the same site.

A second type of "regulatory" nucleotide-binding site, one that is devoid of catalytic activity, has been defined in a number of other nucleotide-binding proteins (242). These regulatory sites are apart from the catalytic site of the enzyme and often reversibly bind more than one nucleotide to the same site. Examples include aspartate carbamolyltransferase (binds ATP as a positive effector and CTP as a negative regulator), and phosphofructokinase (binds ADP as a positive effector, phosphoenol pyruvate as a negative regulator). Other examples include phosphoglycerate kinase, as well as four pyrimidine nucleotide kinases (uridine, thymidine, deoxythymidine, and cytidine kinases). Uridine kinase binds both UTP and CTP to its regulatory site. Similar results were found for thymidine kinase. Each of these enzymes contain both a catalytic

nucleotide-binding site (which requires the ATP- Mg^{2+} complex) and a separate and distinct regulatory site. Thus, whether or not the ATP- Mg^{2+} complex is required in the binding of a nucleotide to the regulatory site of these proteins is difficult to determine.

The consensus sequences of these regulatory sites are separated into two domains, Reg-1 and Reg-2 (242). Reg-1 is responsible for the binding of the base moiety of the nucleotide and thus determines the specificity of the protein-nucleotide binding. The consensus sequences of this domain are considered speculative as they often exhibit a lower specificity for nucleotides than do catalytic nucleotide binding sites. Reg-2 is the domain responsible for outlining the peptide segments that bind the phosphate residues of the bound nucleotide. The consensus sequences in this domain exhibit both variant levels of substitution and spatial arrangement as well. A highly conserved tyrosine residue, whose function is as yet undefined, is found within this domain. The secondary structure of these motifs is yet to be determined.

The amino acid sequence of Glo-II from human liver or *Arabidopsis thaliana* does not possess the conserved sequences that are required for the formation of the catalytic nucleotide-binding site. Furthermore, our studies do not find that the binding of ATP to Glo-II-s requires the presence of Mg^{2+}; nor is the activity / sensitivity of the enzyme affected by any divalent cation tested. Although catalytic nucleotide-binding sites generally bind only one nucleotide, we have shown that four different nucleotides bind to Glo-II. Thus, it is highly unlikely that Glo-II-s is a protein in which a “catalytic” nucleotide-binding site is present.

Although no Reg-1 domain can be identified in Glo-II, it is known that the consensus sequence for this domain is highly variable and may not be distinguishable. The sequence of GLO-II from human liver or *Arabidopsis thaliana* does contain a possible variance of the Reg-2 domain. Three possible Reg-2 nucleotide-binding domains, each of which overlaps the other and utilizes the same conserved tyrosine residue (tyrosine-76), are found between residues 35 – 82 in the amino acid sequences of Glo-II from either source. Each of these sites contains a possible insertion of a valine residue within the consensus sequence. The presence of a regulatory nucleotide-binding site in GLO-II is therefore possible, given the known variances in the consensus sequences of the two domains.

Other similarities in the binding of nucleotides to both Glo-II-s and other enzymes, which are known to possess "regulatory" nucleotide binding sites, are apparent as well. We have shown that Glo-II-s binds more than one nucleotide, as do these regulatory sites. The binding of nucleotides to Glo-II-s occurs at a site apart from the catalytic site of the enzyme, similar to that of nucleotide binding to regulatory sites. Furthermore, the binding of nucleotides to Glo-II-s is apparently not of the "catalytic" type of binding described above, thus the ATP-Mg^{2+} complex may not be required for binding. These regulatory sites are generally found in proteins that form multimers; the existence of multimer forms of Glo-II has been previously reported (181).

Glo-II-i – Preparation from Glo-II-s

Two methods were developed in our laboratory to produce samples of Glo-II-i for comparative studies. The incubation of the Glo-II-s preparations at 12°C consistently resulted in a gradual decrease of the enzyme activity (Figure 13). Coupled with the decrease in activity, the inhibition characteristics of the enzyme preparations also decreased in a parallel manner. Although the time period of incubation for the completion of the process varied from sample to sample, this "slow" process of de-sensitization consistently resulted in an enzyme preparation that exhibited greatly reduced inhibition when assayed in the presence of ATP.

The incubation of the Glo-II-s enzyme in the presence of high concentrations of ATP was shown to cause a similar, but much more rapid decrease in enzyme activity (Figure 14). As with the de-sensitization of Glo-II-s by incubation at 12°C, the inhibition characteristics of the Glo-II-s enzyme decreased in a similar, rapid, manner. Again, the time period of preparation for the completion of this "rapid" process of de-sensitization varied from sample to sample, but the process consistently resulted in an enzyme solution that exhibited greatly reduced inhibition when assayed in the presence of ATP.

Both de-sensitization processes caused some loss of enzyme activity and the near complete loss of the inhibition of the enzyme by ATP. These insensitive enzyme preparations thus obtained were then re-purified to remove any degraded protein, and ATP, from the solutions, and then used for further study. These re-purified preparations were designated as Glo-II-i.

Glo-II-i – Kinetic Properties

Both procedures for the de-sensitization of Glo-II-i result in enzyme preparations that exhibit linear plots over the substrate range tested, as analyzed by the double reciprocal plot method of Lineweaver-Burk (Figures 16, 19). Further, these enzyme preparations exhibit equal Km values for SLG (0.22 mM), a value that is approximately 2.5-fold lower than the value determined for the enzyme activity before de-sensitization (i.e., Glo-II-s, Km = 0.54 mM).

The inhibition of Glo-II-i by ATP is greatly diminished when compared to that of Glo-II-s (Figures 15, 18). At physiological concentrations of ATP (2 mM), inhibition was approximately 15% for the Glo-II-i enzyme. Maximum inhibition of Glo-II-i was approximately 20% at 10 mM ATP. In contrast, the Glo-II-s enzyme was inhibited to a significantly greater extent when assayed in the same manner (> 50% inhibition at 2 mM ATP, approximately 65% inhibition at 10 mM ATP).

Since the inhibition of the insensitive enzyme was low, the use of replots of inhibition data to determine the number of enzyme forms was not practical. Therefore, the presence of more than one form of enzyme in the desensitized samples was determined by Eadie – Scatchard replots of the kinetic data in the absence of inhibitor. This method is capable of detecting the presence of more that one form of an enzyme in a solution by the non-linearity of the plots, if the two forms exhibit different Km values for the substrate used. The linear replots suggest that only one kinetically distinguishable form is predominant after the process of de-sensitization of Glo-II-s (Figures 17, 19).

A significant decrease in the Km value of the enzyme for the substrate occurs along with the loss of sensitivity to nucleotide inhibition when Glo-II-s preparations are subjected to de-sensitization procedures. It is interesting to note that the change in the Km value occurs at different stages in the two de-sensitization processes . The decrease in the Km value occurs during the incubation period of the 12°C-de-sensitization process. For de-sensitization by ATP, the change in the Km value does not occur until after the re-purification step. Thus, it is likely that the rate of de-sensitization of purified Glo-II-s samples observed during incubation at 12°C is greatly accelerated by the presence of ATP during re-purification.

Although the Km value of the enzyme for SLG decreases during de-sensitization, the Vmax of the Glo-II enzyme does not change significantly (Figure 21). Utilizing the different experimental Km values for the two enzyme preparations, the similar Vmax values, and the constant substrate concentration used for the assay (0.25 mM SLG), the calculated initial velocity of the Glo-II-i preparation is 1.63-fold greater than the initial velocity of the Glo-II-s preparation. Thus, the calculated initial velocities for the two enzyme forms suggest that, if no enzyme activity is denatured during the incubation, the transition of Glo-II-s to Glo-II-i should be coupled with an apparent increase in the activity of the enzyme. Therefore, the loss of Glo-II activity observed during the de-sensitization procedures is likely a “real” loss due to the denaturation of some portion of the enzyme present in the original Glo-II-s mixture of enzyme forms. Furthermore, the enzyme form that is denatured during the process is predictably that of the Glo-II-s form of the enzyme; this assumption is based on the observed loss of the inhibitory

characteristics of the Glo-II-s preparation during the process. This assumption is further supported by the linearity of the Eadie-Scatchard replots of the kinetic data collected from the Glo-II-i enzyme that suggest the presence of only one form of the enzyme in the solution after de-sensitization.

If the maximum velocity of the Glo-II-s enzyme does not change when transformed to the Glo-II-i form, then, by definition, the value for k_{cat} has not changed. Thus, the higher Km value of the original Glo-II-s preparation for SLG is apparently due to a change in the dissociation constant (Ks). This could result from a decrease in the rate of formation of the encounter complex (ES) between the enzyme and the substrate, an increase in the rate of dissociation of the ES complex to free enzyme and substrate, or a combination of both.

Glo-II-s versus Glo-II-i – Structural and / or Conformational Differences

A series of studies was conducted to determine if the different kinetic and inhibition characteristics of Glo-II-s and Glo-II-i were due to structural or conformational changes in the enzyme. No detectable differences in the relative molecular weights, as determined by SDS-PAGE (Figure 1), or of molecular dimensions of the enzyme, as determined by gel filtration (Figure 22), were observed. Neither did the presence of ATP cause any detectable differences in the gel-filtration profiles of any of the enzyme forms tested (Figure 23). Circular dichroic analysis of the two forms of the enzyme revealed no significant differences in the spectra, suggesting that no major changes in secondary structure occur when Glo-II-s is converted to Glo-II-i (Figure 24). Both forms of the

enzyme (Glo-II-s or Glo-II-i) exhibit nearly identical temperature denaturation curves as analyzed by the circular dichroism of the two proteins at 220 nm over a temperature range of 25°C to 90°C (Figure 25). The high temperature necessary for the complete loss of the secondary structure of the protein (> 80°C) suggest that Glo-II is a compact, relatively stable protein structure. The similarities in the temperature denaturation curves also suggest that any structural differences between Glo-II-s and Glo-II-i are not likely due to significant differences in the folding characteristics of the protein.

Since no apparent differences in the structures of the two forms of the enzyme were detectable by the methods used above, we assumed that any structural differences must be due to small micro-environmental changes in protein conformation. This assumption was supported by the notable differences in the fluorescent emission spectra of the tryptophan residues of Glo-II-i when compared to that of Glo-II-s (Figure 26). This assumption was even further supported by the differences in the anisotropic fluorescent emission spectra of the tryptophan residues of the two protein preparations (Figure 27). Therefore, our data suggest that structural differences between Glo-II-s to Glo-II-i, the loss of the inhibitory characteristics of the enzyme, and the change in the value of the Km of the enzyme are apparently due to small micro-environmental changes in protein conformation.

Glo-II-s; Chemical Modification Studies

Chemical modifications of Glo-II-s were conducted to determine if the modification of those residues tested effected the inhibition characteristics of the enzyme. Modification of the histidine or arginine residues of Glo-II-s caused a loss of activity, similar to that previously reported, but no effect on the inhibition of the enzyme activity by ATP was observed (Figures 28, 29). Modification of serine residues had no effect on enzyme activity, as found in previous reports; no effect on the inhibition of the enzyme activity by ATP was observed. Studies on the effect of metal ions (including Mg^{2+}) or chelation agents had no effect on either the activity or sensitivity of Glo-II-s as well. Taken together, these data suggest that Glo-II-s responds in a manner similar to that of previously reported for Glo-II with respect to the chemical modification of certain amino acid residues of the protein. Furthermore, the modification of these residues (or the addition of ions or chelation agents) does not affect the inhibition characteristics of Glo-II-s.

Re-sensitization of Glo-II-i

The chemical modification of Glo-II-s with a variety of sulfhydryl group reagents had no effect on either the activity or the sensitivity of the enzyme (Figure 30). Furthermore, no free sulfhydryl groups in the protein could be detected. Finally, no protection from the loss of activity and / or sensitivity was furnished by the addition of reducing agents such as DTT. These results, taken together, suggested that the sulfhydryl

groups of the protein are not involved in the activity and / or sensitivity of the enzyme. Furthermore, these data suggested that the cysteine sulfhydryl groups of Glo-II-s exist in the form of disulfide bonds.

DTA is a reagent that has been well documented for its ability to efficiently cleave disulfide bonds, reducing them into free sulfhydryl groups (241). The efficiency of the reagent in this reaction is due to its low pKa (7.8) for the formation of the active species, the thiolate anion (241). Other reagents capable of cleaving such bonds (e.g., DTT) are far less efficient in this reaction due to the characteristically high pKa values (> 9) for the formation of the active thiolate anion (241). Furthermore, DTA is considered far more specific than other reagents used to cleave disulfide bonds (mercury, cadmium, or arsenic) (241). Therefore, DTA was employed as a reagent to study the effect of the reduction of disulfide bonds on the activity and sensitivity of Glo-II-s or Glo-II-i.

Modification of Glo-II-i by DTA results in two stages of modification; an initial stage involving re-sensitization followed by a stage involving loss of enzyme activity (Figures 31, 32). The re-sensitization stage is thus characterized by the significant increase in the inhibition of the enzyme by ATP. No apparent change in the activity of the enzyme, when assayed at high substrate levels is detectable during this stage. Once the re-sensitization of the enzyme appears complete, the second stage is characterized by the gradual loss of enzyme activity. No further change in the inhibition characteristics of the enzyme is observed during this stage. Long incubation periods result in the complete loss of enzyme activity.

Treatment of Glo-II-s with DTA results in a one-stage process that is very similar to the gradual decreased enzyme activity observed during the incubation of Glo-II-i with DTA (Figures 3, 34). Only very small increases in the inhibition characteristics of Glo-II-s were, at times, observed, while longer incubation periods invariably cause the complete loss of enzyme activity.

Our findings show that the treatment of Glo-II-i with DTA results in an enzyme form that is sensitive to inhibition by ATP. If one assumes that one (or more) disulfide bond(s) has (or have) been reduced in this process, then the inhibition of Glo-II activity by ATP requires the presence of at least two free sulfhydryl groups. If the proposed free sulfhydryl groups in Glo-II-s were, by some process, susceptible to oxidation, upon formation of the resulting disulfide bond(s) Glo-II-s would thus be converted back to Glo-II-i. The data also suggest if all the disulfide bonds in the protein are reduced (long incubation periods), complete loss of activity results.

The results from the studies on the incubation of Glo-II with DTA are contrary to those involving other sulfhydryl group reagents. It may be that the free –SH groups in Glo-II-i are inaccessible to the reagents used in these experiments. The –SH reagents used may not be sufficiently reactive to effect modification. Although the nature of the cysteine residues in Glo-II is still unclear, it is obvious that modification of the enzyme by this reagent causes the re-sensitization of the enzyme.

Glo-II-s (Obtained by DTA treatment of Glo-II-i) – Kinetic Characteristics

The DTA-treated enzyme exhibits kinetic characteristics similar to that of Glo-II-s. The Km for SLG was found to be 0.6 mM and is comparable to the Km for the originally purified Glo-II-s preparation, 0.54 mM (Figure 35). The major difference in the kinetic characteristics of the two enzyme preparations came from analysis of the replots of the kinetic data (Figures 36, 37). Replots show that the parabolic curve of the original mixture of enzyme forms in the Glo-II-s preparation is no longer evident. The data support hyperbolic, hyperbolic, partial non-competitive inhibition, and suggest that only one form is now present in the sample (238). This suggestion was further supported by the linearity of the Eadie-Schatchard replot of kinetic data from subsequent experiments with Glo-II-s (Figure 38). It is assumed under these conditions that an active ESI complex is formed that is still capable of producing product, but at a reduced rate. It is thus apparent that the enzyme form that is predominant in these preparations is now the nucleotide sensitive form of the enzyme. Since the re-sensitized enzyme was observed to be highly unstable when attempting to remove the DTA by re-purification, yields were extremely poor. The value for the specific activity of this preparation was not considered reliable.

Physiological Implications

As stated previously, quantitative kinetic models predict that Glo-II is a near optimal catalyst for the hydrolysis of SLG (186, 189, 223). These models predict that

Glo-II activity, under physiological conditions with substrate concentrations much lower than the Km value for the substrate, is significantly limited by the diffusion controlled collision of the enzyme with the substrate, a major criteria for the optimality of an enzymatic reaction. This type of enzymatic activity would be indicative of an enzyme that operates as the exit step of a detoxification system. From calculations based on known values for the kinetic parameters and the cellular concentrations of Glo-II, the steady-state concentrations of SLG in a cell must be held at low nanomolar concentrations (2 nM) in order for these models to be valid (223).

Another model for the catalysis of SLG by Glo-II is derived from *in vivo* studies in which the predicted optimality of the enzymatic reaction is significantly decreased. These studies indicate that the steady state concentration of SLG found in cells (15 μM – 20 μM) is three orders of magnitude greater than that predicted by the quantitative kinetic model (218). Furthermore, the Glo-II activity of tissues has been reported to exhibit up to 10^4 increases in activity upon cell lysis (243). Finally, *in vivo* studies have shown that the Glo-II activity of certain differentiating or activated tissues exhibit changes in such a manner that a putative, but as yet unidentified non-competitive effector of Glo-II activity exists within these cells (73, 187).

A great deal of research has been directed toward the effect of certain disease states on the flux of methylglyoxal through the glyoxalase system. Disruption of glyoxalase activity, resulting in changes in the efficiency of the removal of toxic methylglyoxal from tissues, has been observed in both cancerous (211) as well as diabetic tissue (220). In part, these observed changes in system flux may be attributed to changes

in the concentration of the initial substrate for the system, methylglyoxal. Alternatively, studies have predicted that the resulting buildup of glyoxalase metabolites in disease states is, at least in part, due to dramatic changes in the activity of the glyoxalase enzymes (211, 220). In particular, a reduction of the catalytic efficiency of the exit step of the system, that reaction catalyzed by the Glo-II reaction has been reported (211).

We have demonstrated that two forms of Glo-II are purified by the method developed in our laboratory. One of these forms, Glo-II-i, appears as a more optimal catalyst for the metabolism of SLG due to its greater affinity for the substrate. As a consequence of the increased substrate affinity, the enzyme would exhibit a somewhat higher enzymatic velocity (1.8 fold) with respect to the hydrolysis of the substrate when compared to that of the Glo-II-s form of the enzyme. Furthermore, ATP does not significantly inhibit the Glo-II-i form of the enzyme.

In contrast to the Glo-II-i form, the Glo-II-s form appears as a much more inefficient enzyme in its role of SLG catalysis. It has a significantly reduced affinity for the substrate, and furthermore, its catalytic activity, *in vivo*, would be greatly decreased, since it would be constantly bathed with inhibitory, cytosolic levels of ATP (2 - 4 mM).

The existence of two forms of the Glo-II, *in vivo*, suggests three interesting case scenarios. Each of these cases can be theoretically true. Each case also lends itself to predictive explanations for the aforementioned enigmas, described above, that glyoxalase researchers are faced with today.

Case 1:only Glo-II-i exists in the cell: This scenario would be in agreement with the predictions of the quantitative kinetic models of the glyoxalase system which predict that the Glo-II reaction is acting at near optimal efficiency. Our studies suggest that the k_{cat} of the two forms of the enzyme is the same. Furthermore, the Km value determined for the Glo-II-i enzyme (0.22 mM) is in very close agreement with the value used in these models. Since the kinetic model is based on the values calculated for k_{cat} / K_M for the enzyme, then *in vivo*, the enzyme is significantly limited by the diffusion controlled encounter between the substrate and enzyme and acts at nearly optimal efficiency.

This scenario provides no explanation for the significantly higher levels of SLG actually found in cells, nor does it provide any explanation for the possibility of the existence of a non-competitive inhibitor found in cells as predicted by the *in vivo* studies. Further, it doesn't provide for any explanation for the changes in Glo-II activities that have been reported in diabetic or cancerous tissues. Finally, the Glo-II-s form of the enzyme would be relegated to being an artifact of the purification method, a suggestion that seems unlikely due to the inhibition of Glo-II activity by purine nucleotides found in crude cytosolic fractions from a variety of life forms.

Case 2; only Glo-II-s exists in the cell: This scenario is in close agreement with the *in vivo* predictions that state that Glo-II does not always act in an optimally efficient manner in cells. It is in full agreement with the prediction of the existence of a non-competitive inhibitor of Glo-II activity in cells. The reduced affinity of the enzyme for the substrate as well as the reduced velocity of an enzyme that is constantly inhibited by a

physiological non-competitive inhibitor could, therefore, be responsible for the increased concentrations of SLG found *in vivo*.

This scenario would relegate the Glo-II-i form of the enzyme to that of an artifact of the traditional purification methods previously reported in the literature. This enzyme form was most probably that utilized by researchers to develop the quantitative kinetic models, which predict that Glo-II in cells is acting in a near optimal manner. The possibility of the presence of a regulatory nucleotide-binding site in Glo-II-s provides a putative explanation for the changes in Glo-II activity during the aforementioned disease states.

Case 3 – both forms exist in the cell and are interconvertible: The existence of two forms of Glo-II in a cell, that are interconvertible, provides still another scenario to explain the discrepancies found in comparisons between the *in vitro* and *in vivo* studies. The kinetic models, which describe an optimally efficient Glo-II, can be explained by noting that the studies leading to these models did not account for, nor did they consider, the existence of a Glo-II-s form, which has been demonstrated to exist in the present study. Discrepancies, such as the increased levels of SLG found *in vivo*, the proposed existence of a non-competitive inhibitor in subject cells, and the less than optimal efficiency of the Glo-II reaction found, may be attributed to the existence of the Glo-II-s form of the enzyme in cells. Finally, the changes in Glo-II activity reported to occur in diseased or stressed tissues (e.g. diabetic or cancerous) may be attributed (at least partially) to changes in the ratio of the two enzyme forms in cells. At one extreme, if all the enzyme

exists as Glo-II-i, and the intracellular SLG concentrate is 20 μM, the rate of the enzymatic reaction can be calculated to be almost 10-fold higher than when all the enzyme exists as Glo-II-s.

Of course, the predictions above are entirely hypothetical and are based on considerable speculation. Other factors not yet discovered or understood, could be responsible for the discrepancies found in comparisons between *in vitro* and *in vivo* studies of the glyoxalase system. At this point, the physiological implications of this work remain unclear. It is interesting to envision that the ratio of two interconvertable forms of the enzyme could be dependent upon stress conditions (e.g., oxidative stress). The possibility of the presence of a regulatory nucleotide-binding site in Glo-II-s is of even more interest. Thus, the interconversion between the enzyme forms or the regulation of Glo-II activity by nucleotides would be determined by factors that allow an optimal response to the needs of the cell under varying conditions.

The central outcome of this work is that Glo-II can exist in two kinetically distinguishable forms that can be co-purified. More importantly, we have provided the tools to consistently separate or modify this co-purified pair of enzyme forms such that only one form of the enzyme is predominant in the preparation and can be further studied. Finally, and of paramount importance, we have drawn attention to the finding that the inhibition of the Glo-II activity of cytosolic tissue extracts by nucleotides is an inherent characteristic of one of the two forms of the enzyme. The physiological implications of these findings, although speculative, warrant further study.

APPENDIX A

TABLES

Table I

Content of Glo-I in Adult Human Tissues

Tissue	Radioimmunoassay ($\mu g\ mL^{-1}$)	Specific Activity ($\mu mol\ min^{-1}\ mg^{-1}$)
Adipose	0.3	0.03
Adrenal	2.3	0.08
Aorta	0.2	0.07
Brain	1.3	0.11
Heart	1.4	0.08
Kidney	1.8	0.11
Liver	2.5	0.03
Lung	1.5	0.12
Diaphragm	1.6	0.04
Small Intestine	1.3	0.04
Spleen	2.5	0.08

Data from Reference 36.

Table II

Molecular Characteristics of Glo- I

Molecular Characteristic	Human (Adult)	Bacterial (*P. putida*)	Yeast (*S.cerevisiae*)
Number of Subunits	2	1	1
Metal Content	1 zinc per subunit	1 zinc	1 zinc
Molecular Mass	46,000	19,500	30,000
Isoelectric point	4.8 – 5.1	4.0	7.0
Polymorphism	1-1, 1-2, 2-2	?	?
Km for methylglyoxal (mM)	0.13	3.50	0.53
Molecular Activity (K_{cat}) (min^{-1})	$7\text{-}11 \times 10^4$	?	3.5×10^4

Data from Ref. 89, 106, 108, 110, 114, 115.

Table III

Tissue Distribution of Glo-II Activity in Vertebrates (μmol / min^{-1} mg^{-1})

Vertabrate	Brain	Heart	Kidney	Liver	Muscle	Pancreas	Spleen
Chick	187	93	938	270	70	50	77
Guniea Pig	229	165	374	423	82	179	58
Hamster	64	165	177	246	45	84	27
Human	245	80	99	108	108	49	0
Mouse	68	9	104	124	21	71	27
Ox	69	63	159	529	79	64	34
Pig	80	35	54	163	30	35	24
Pigeon	187	62	311	376	29	93	90
Rabbit	86	55	129	149	52	39	23
Rat	77	19	107	93	13	74	36

Data from Reference

Table IV

Molecular and Kinetic Characteristics of Glo-II

Source	Mol. Wt. (kDa)	Isoelectric Point	Specific Act. (I.U./ mg)	Km (μM)	# of forms
Cytosolic Form					
Human Liver	22.9	8.35	822	146	1
Mouse Liver	29.5	8.1	920	270	1
Rat Liver	24	8, 7.4	----	351	2
Bovine Calf Brain	23	8, 7.4	----	351	2
Rat Brain	24	8, 7.4	----	351	2
Rat Erythrocyte	21.9	------	773	----	-
Bact. (*H. mrakii*)	24	4	----	80	2
Fungal (*Cand. alb*.)	29	6	----	142	1
Yeast (*S. cerevisiae*)	19	----	----	----	--
Plant (*Zea mays*)	26	4.5	65	137	1
Plant (*Aloe vera*)	27	4.7, 4.8, 5.0	536	150	3
Plant (*Spin.oleracea*)	26	5.3, 5.8, 6.2	----	240, 150, 200	3
Mitochondrial Form					
(bovine calf liver)	23-24	6.3 - 7.9	134	290	5
(*Spinacea oleracea*)	26	4.8	----	170	1

Data from References 168, 169, 171, 175 – 180, 182, 206

Table V

Substrate Specificity of Human Glo-II from Human Liver

Substrate	V max (relative)	Km (μM)
S-Lactoylglutathione	100	190
S-Glycerylglutathione	62	109
S-Acetoacetylglutathione	56	295
S-Glycolylglutathione	39	70
S-Formylglutathione	38	153
S-Succinylglutathione	29	200
S-Propionylglutathione	14	213
S-Acetylglutathione	9	266
S-Mandeloylglutathione	5	16

Data from Reference 168

Table VI

Thiocarbonate / Carbamate Derivatives of GSH as Inhibitors of Glo-II

Inhibitor	Glo-II Source	K_I (μM)	IC_{50} (μM) ([SLG] = 0.4 mM)
S – (p – chlorophenacyl) glutathione	*Zea Mays*	630	----
	Python molurus	400	----
Carbobenzoxyglutathione	Rat Liver	65	165
	Zea mays	26	----
	Candida albicans	48	----
N, S-biscarbobenzoxyglutathione	*Aloe vera*	8	----
o-Nitrocabobenzoxyglutathione	Rat Liver	15	20
m-Nitrocabobenzoxyglutathione	Rat Liver	9	20
p-Nitrocabobenzoxyglutathione	Rat Liver	6.5	20
	Zea mays	5.8	----
S-Fluorenylmethoxycarbonylglutathione	*Aloe vera*	4.6	----
N, S- Fluorenylmethoxycarbonylglutathione	Bovine Calf Liver	0.75	2.5

Data from Reference 25.

Table VII

The Widespread Presence of Inhibition of Glo-II Activity by Nucleotides

SOURCE	GENUS	SPECIES	ORGAN	%Inhibition
Mammalian	Bovine	Cow	Liver	> 40
			Kidney	> 40
			Brain	> 40
	Porcine	Pig	Liver	> 40
			Kidney	> 40
	Murine	Rat	Liver	> 40
		Mouse	Liver	> 40
Plant	Morus	Mulberry	Arial	> 30
	Brassica	Kale	Arial	> 30
Fish	Silurid	Catfish	Liver	> 30
Bacteria	*E. Coli*	Aerobic	complete medium	< 5
		Aerobic	incomplete medium	< 5
		Anaerobic	complete medium	< 5
		Anaerobic	incomplete medium	> 20

TABLE VIII

Purification of Glo-II-S

STEP	Volume (mL)	Total Activity (U.)	Total Protein (mg)	Specific Activity (U. / mg)	Overall Yield (%)	Fold Purif.
Cytosolic Fraction	108	9024	3564	2.53	100	1
Acetone Fraction	98	4895	1192	4.11	54	1.6
Affinity Chromatography	1	3610	2.33	1549	40	612

APPENDIX B

LEDGENDS AND FIGURES

Figure 1; SDS-PAGE of Glo-II Preparations

Analysis of the purity of Glo-II preparations was carried out by SDS-PAGE by the method of Laemmli (231), using a Bio-Rad mini-gel apparatus, with a 3% acrylamide stacking gel and a 15% acrylamide resolving gel (see Methods section). Determination of the molecular mass of the enzyme was done by comparison of the relative mobility of the protein to those of a set of molecular mass standards purchased from Sigma. Detection of the protein was done by silver staining (233). The amount of protein used was 10 ng. Lanes are identified as lanes 1-5 from left to right. Lane 1 contains standard molecular mass proteins in descending order from top to bottom and include (in order):

Bovine Serum Albumin (66 kDa)

Glutamic Dehydrogenase (55 kDa)

Ovalbumin (45 kDa)

Carbonic Anhydrase (29 kDa)

Trypsinogen (24 kDa)

Trypsin Inhibitor (20 kDa)

α-Lactalbumin.

Lane 2 contains Glo-II-s.

Lane 3 contains Glo-II-i de-sensitized by incubation at 12°C.

Lane 4 contains Glo-II-i de-sensitized by incubation with ATP.

Lane 5 contains Glo-II-s re-sensitized by incubation with DTA.

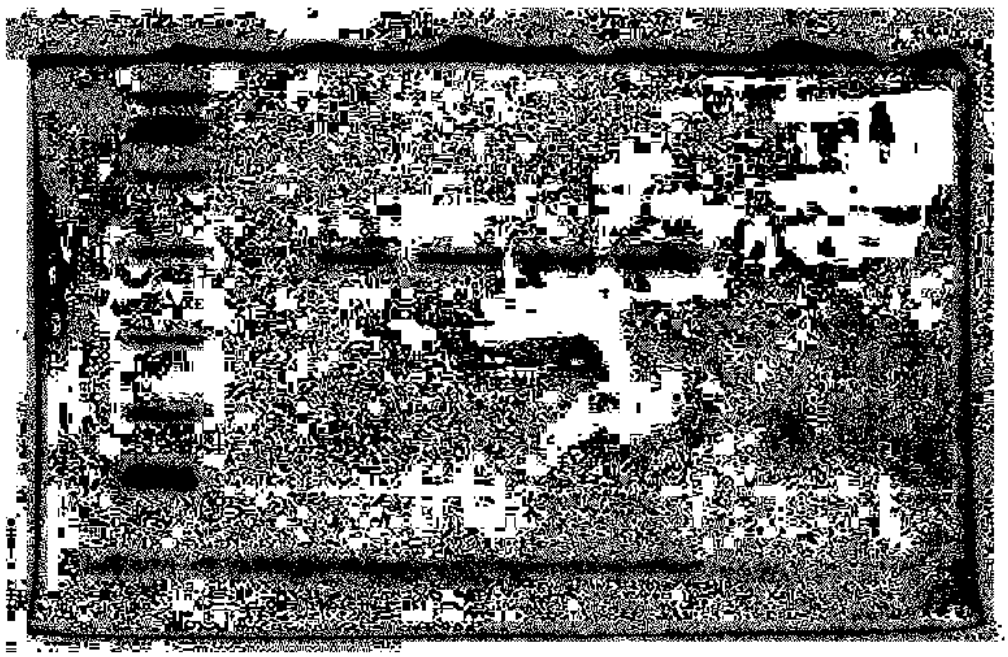


Figure 2; Temperature Stability of Purified Glo-II-s

Glo-II-s (2.5 U / ml) was incubated at various temperatures and aliquots were removed, diluted, and assayed periodically for activity. Assays were conducted as described in Methods section. SLG concentration in the assay was 0.25 mM. Data are recorded as the percent activity of the sample tested relative to the activity of the sample at zero time.

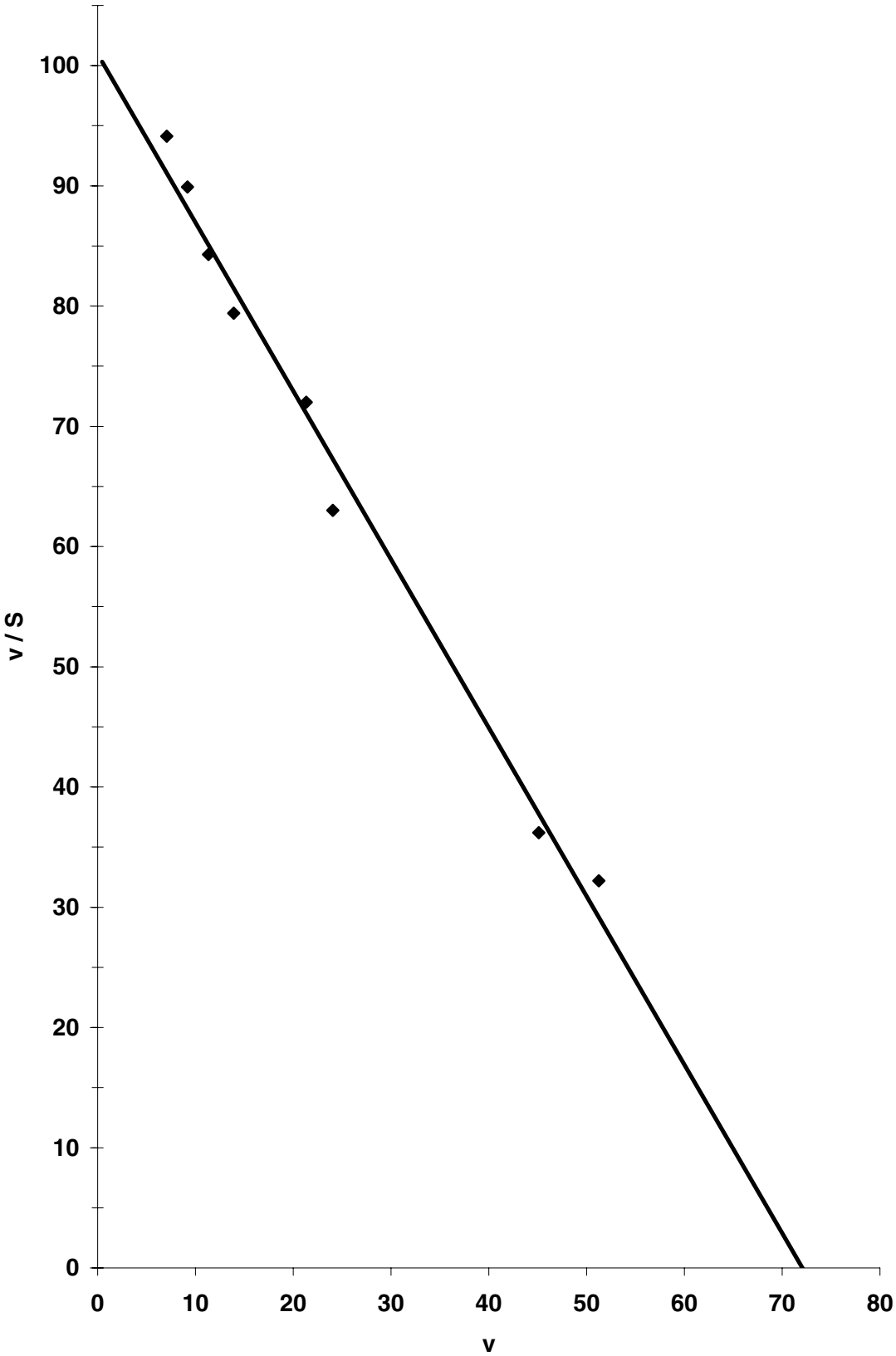

100
90
80
70
60
50
40
30
20
10
0
v / S
0
10
20
30
40
50
60
70
80
v

Figure 3; The Stability of Purified Glo-II-s with Respect to pH

Aliquots of Glo-II-s (2.5 U / ml) were incubated at pH values from 4 to10 for 5 min. at 1°C in a buffer containing a combination of 40 mM citrate, 40 mM TES, and 40 mM TRIS (see Methods section). Aliquots were removed, diluted 20-fold into buffer A, and then assayed for activity. Data were recorded as the percent activity of the enzyme after incubation relative to that of a fresh dilution of an aliquot of the enzyme that had been incubated in buffer A, 1°C. Assays were conducted as described in the Methods section. The SLG concentration in the assay was 0.25 mM.

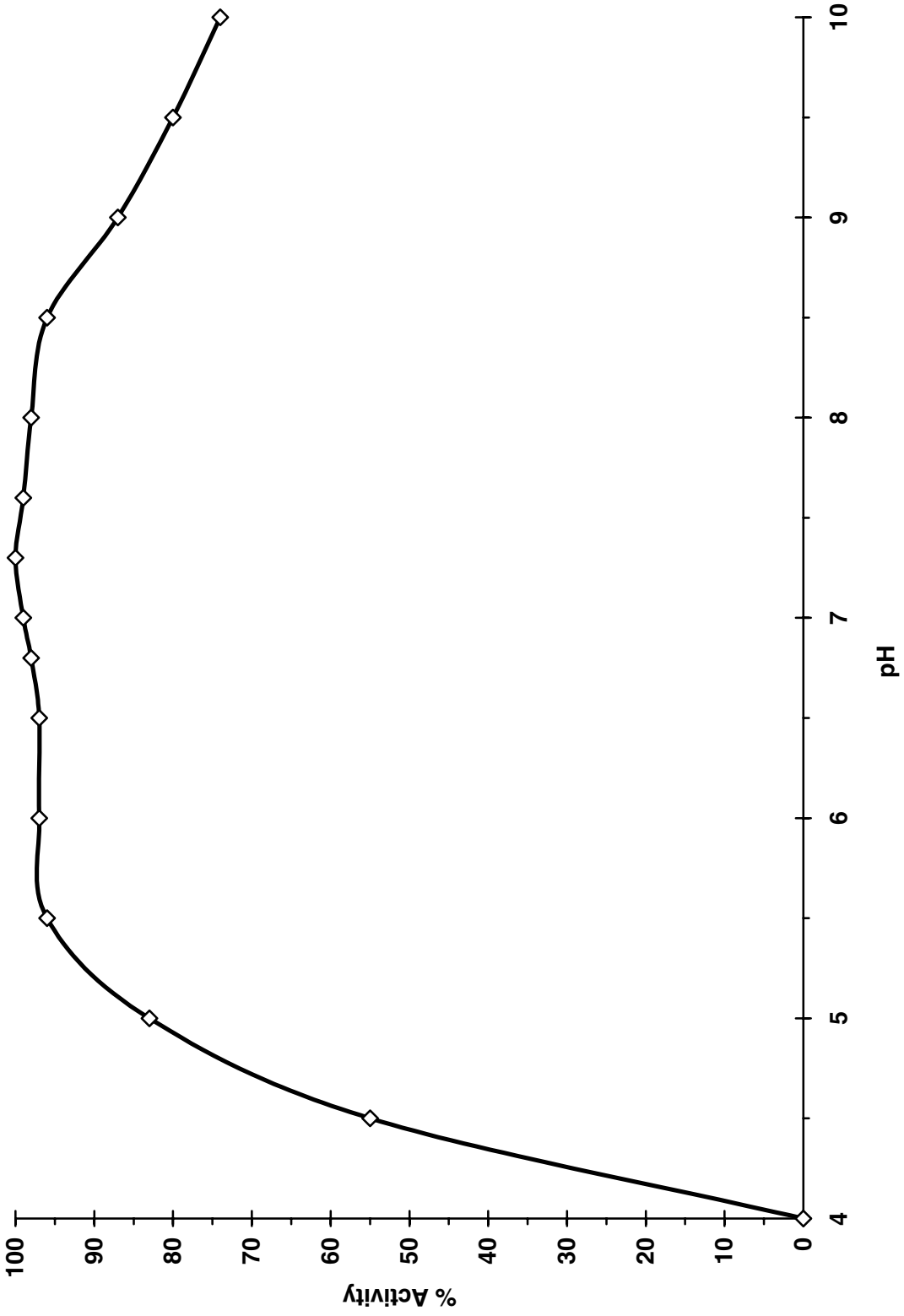

% Activity
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

Figure 4 ; Profile of Glo-II-s Activity and Inhibition by ATP with Respect to pH

Glo-II-s was assayed at pH 5.5-8.5 in a buffer containing a combination of 40 mM citrate, 40 mM TES, 40 mM TRIS, 0.4 mM SLG in 1 ml total volume for 1 min. at 25°C. The reaction was started by the addition of substrate. The reaction was then quenched by the addition of 300 μL of 1N HCL, and then incubated for 5 min.. The pH of the solutions was brought to 7.3 by the addition of 2.7 ml of a 1 M TES buffer, pH 7.3, containing 0.8 mM DTNB. For all samples a blank with no enzyme present was treated similarly. The absorbance of each sample was then read at 412 nm against the appropriate blank. All assays and blanks were then repeated in the same manner with the addition of 2 mM ATP. Data were recorded as the change in μmol of GSH produced from SLG per min.

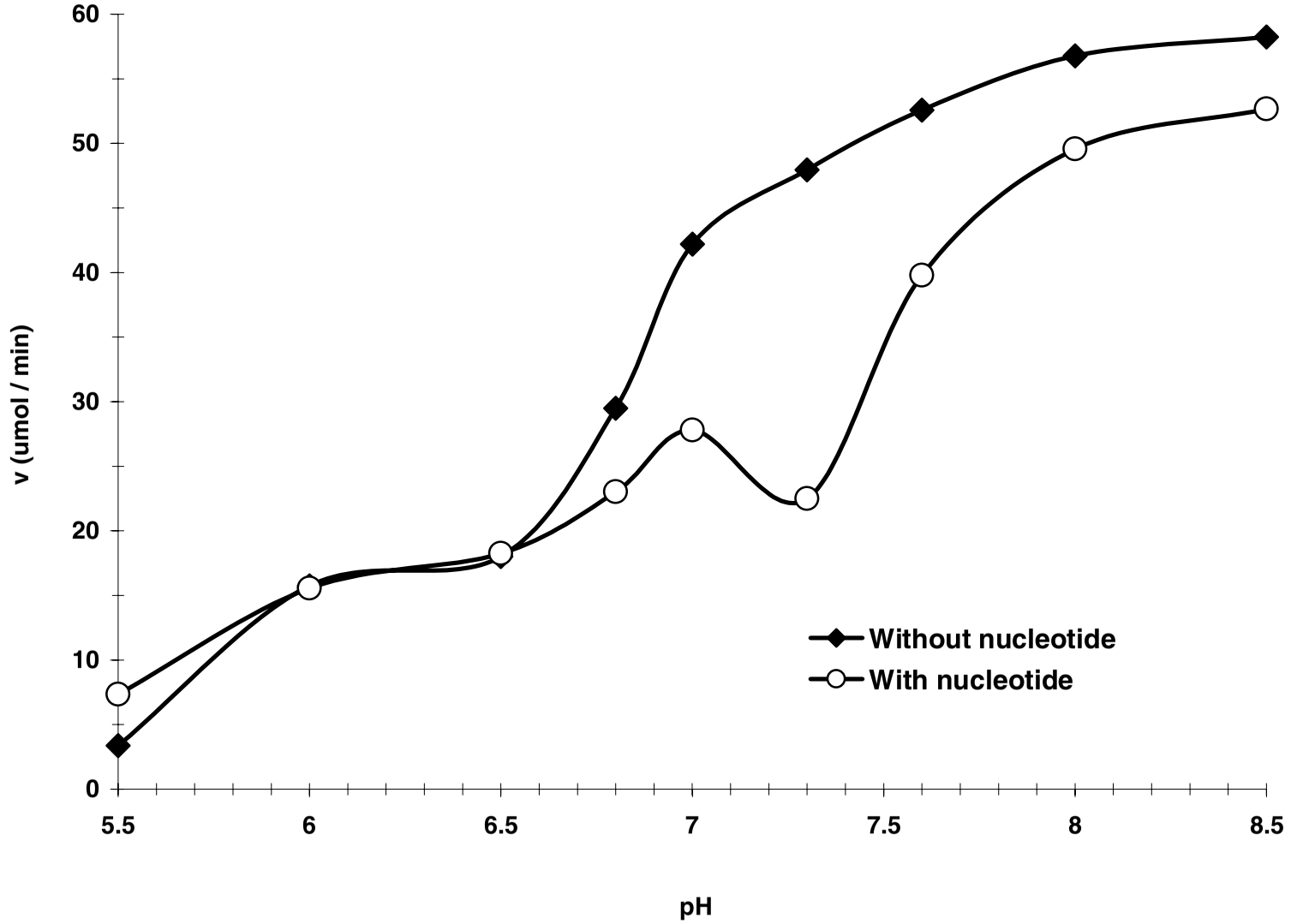

60
50
40
30
20
10
0
v (umol / min)
5.5
6
6.5
7
7.5
8
8.5
pH
Without nucleotide
With nucleotide

Figure 5; Profile of Nucleotide Inhibition of Glo-II-s with Respect to pH

The data from Figure 4 were used to construct the plot. The percent activity was calculated from the ratio of the activity of the enzyme in the presence of nucleotide to that of the enzyme in the absence of nucleotide.

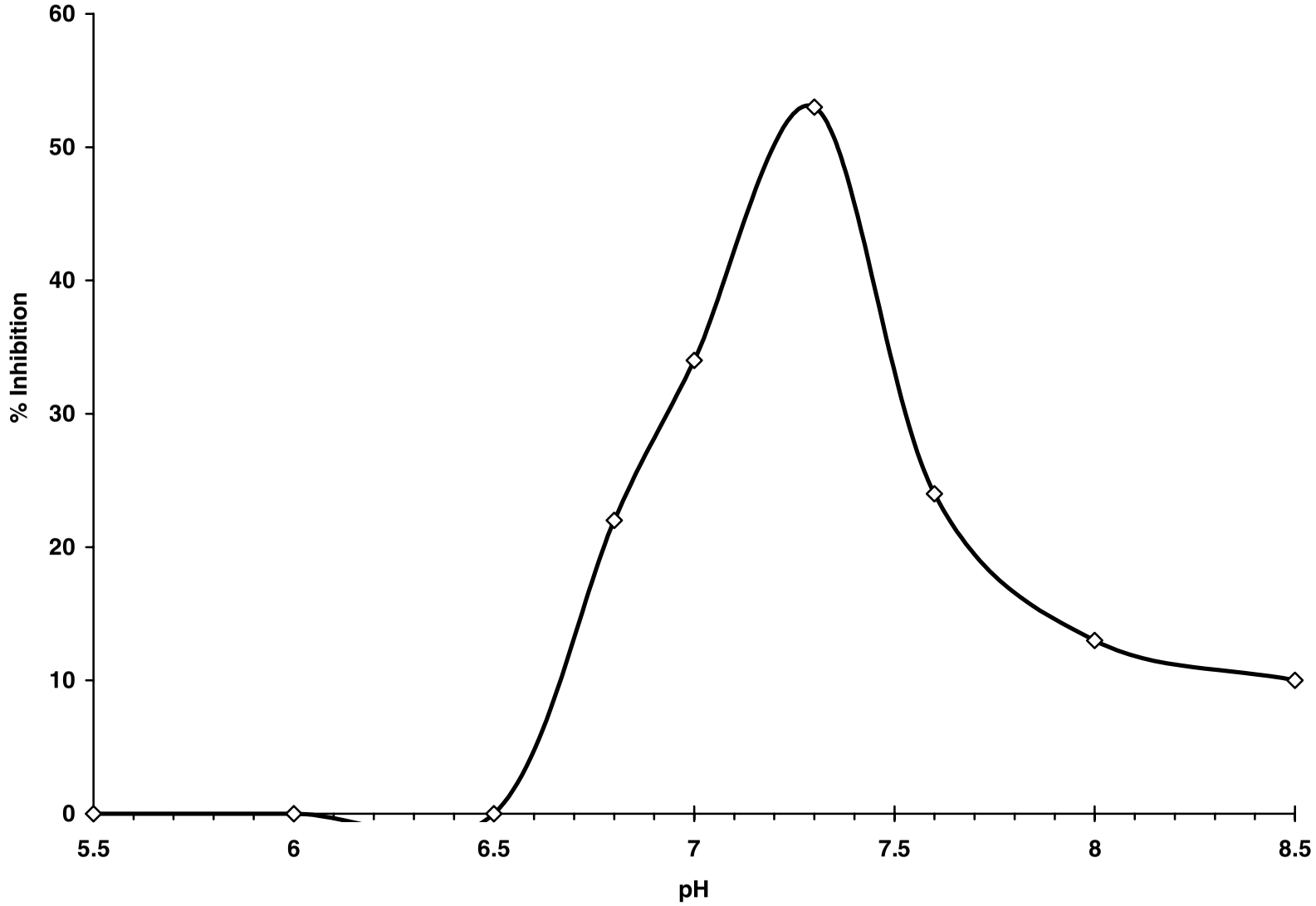

60
50
40
30
20
10
0
% Inhibition
5.5
6
6.5
7
7.5
8
8.5
pH

Figure 6; Inhibition of the Purified Glo-II-s Activity by Nucleotides

Purified Glo-II-s was assayed in the presence of a various nucleotides. The data were then compared to the uninhibited activity (no nucleotide present) of the enzyme. Assays were conducted as described in the Methods section. Substrate concentration in the assays was at 0.25 mM SLG. Nucleotide concentration was 2 mM. The percent inhibitions were calculated from the ratio of the inhibited activity to the uninhibited activity. All samples were assayed in triplicate and averaged.

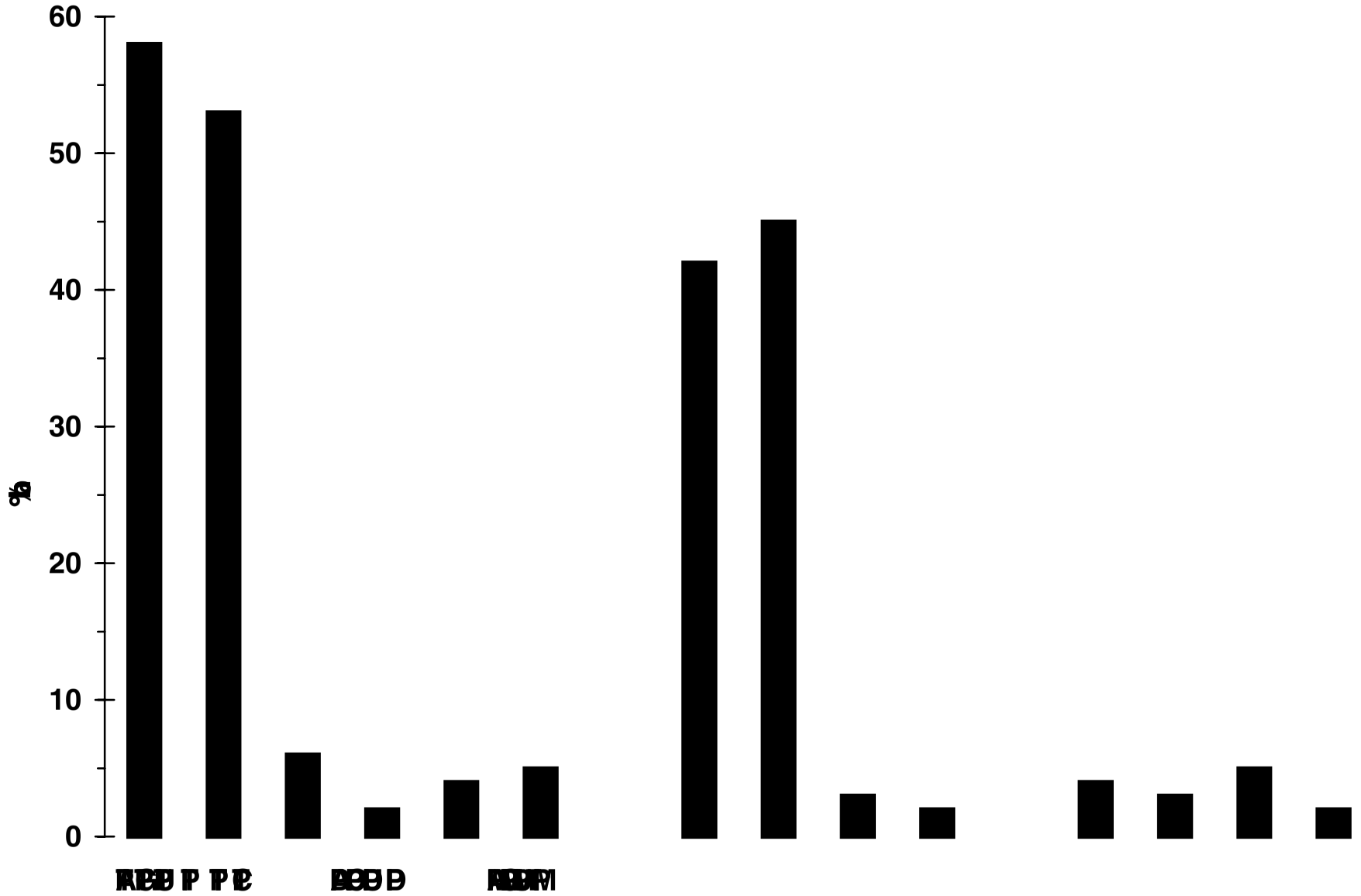

60
50
40
30
20
10
0

Figure 7; Inhibition of Purified Glo-II-s by Nucleotides under Pre-incubation Conditions

Samples of concentrated Glo-II-s (20 U / ml) were incubated for 1 hr. at 12^{O}C in the presence of nucleotide (1.4 mM), then diluted (1-500) into the assay medium such that the residual nucleotide concentration from the incubation was 2.8 μM (a non-inhibitory concentration). A control with no nucleotide added was treated similarly. Assays were conducted at 0.25 mM SLG as described in the Methods section. Data were recorded as the percent activity of the enzyme relative to that of the enzyme at time zero and shown in open bars in the histogram. Assays were also conducted using 0.25 mM SLG in the presence of 1 mM ATP to determine if any change in the extent of inhibition by ATP had occurred. Data were recorded as percent inhibition relative to that of the enzyme assayed in the absence of ATP and shown in closed bars in the histogram. All samples were assayed in triplicate and the averaged.

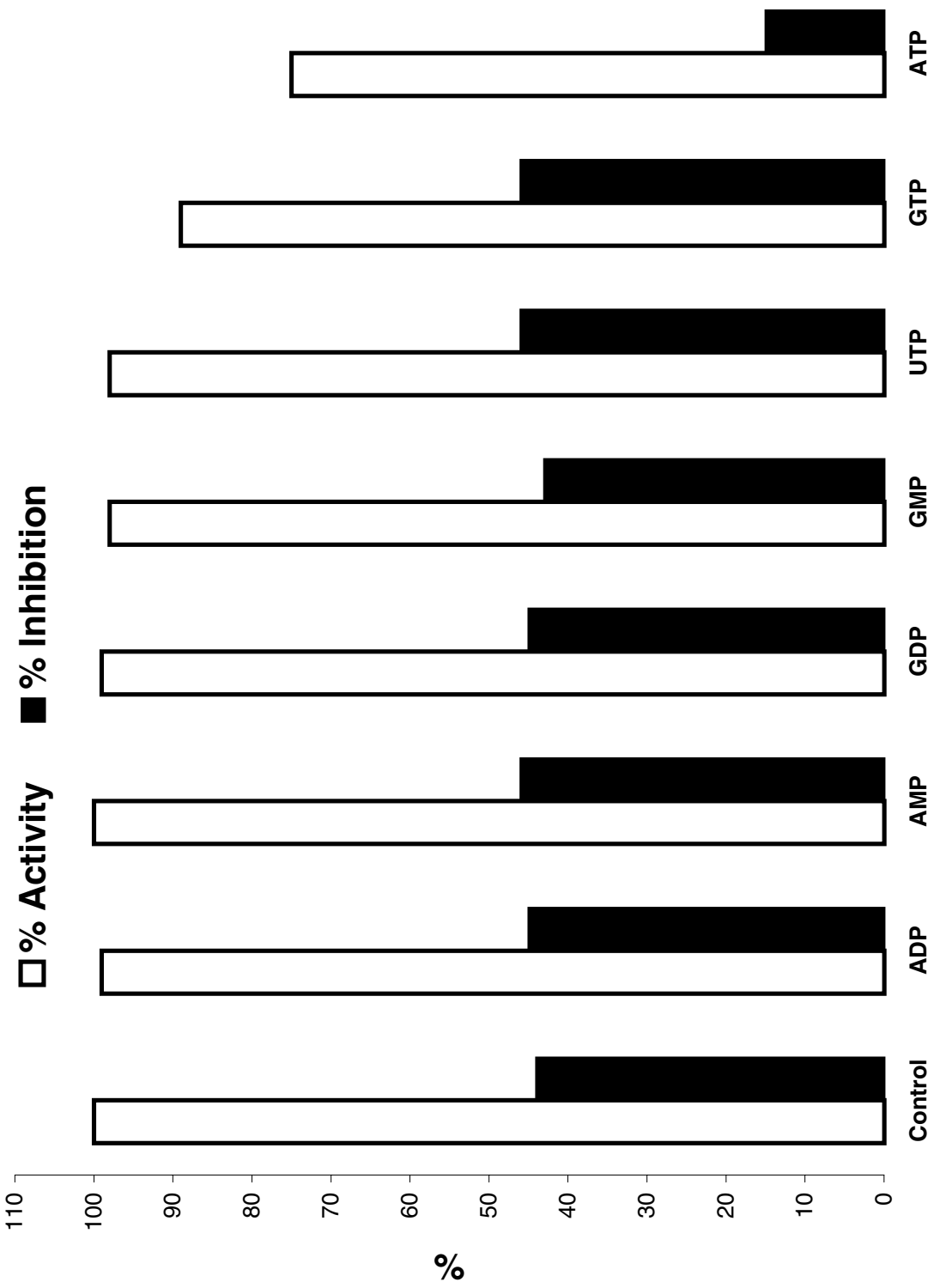

% Activity
% Inhibition
Control
ADP
AMP
GDP
GMP
UTP
GTP
ATP
110
100
90
80
70
60
50
40
30
20
10
0
%

Figure 8; Percent Inhibition of Glo-II-s by ATP

Samples of concentrated Glo-II-s were assayed at varying levels of ATP (0 to 10 mM). All assays were conducted at 0.25 mM SLG as described in the Methods section. Data were recorded as the percent inhibition of the enzyme activity relative to that of the uninhibited enzyme.

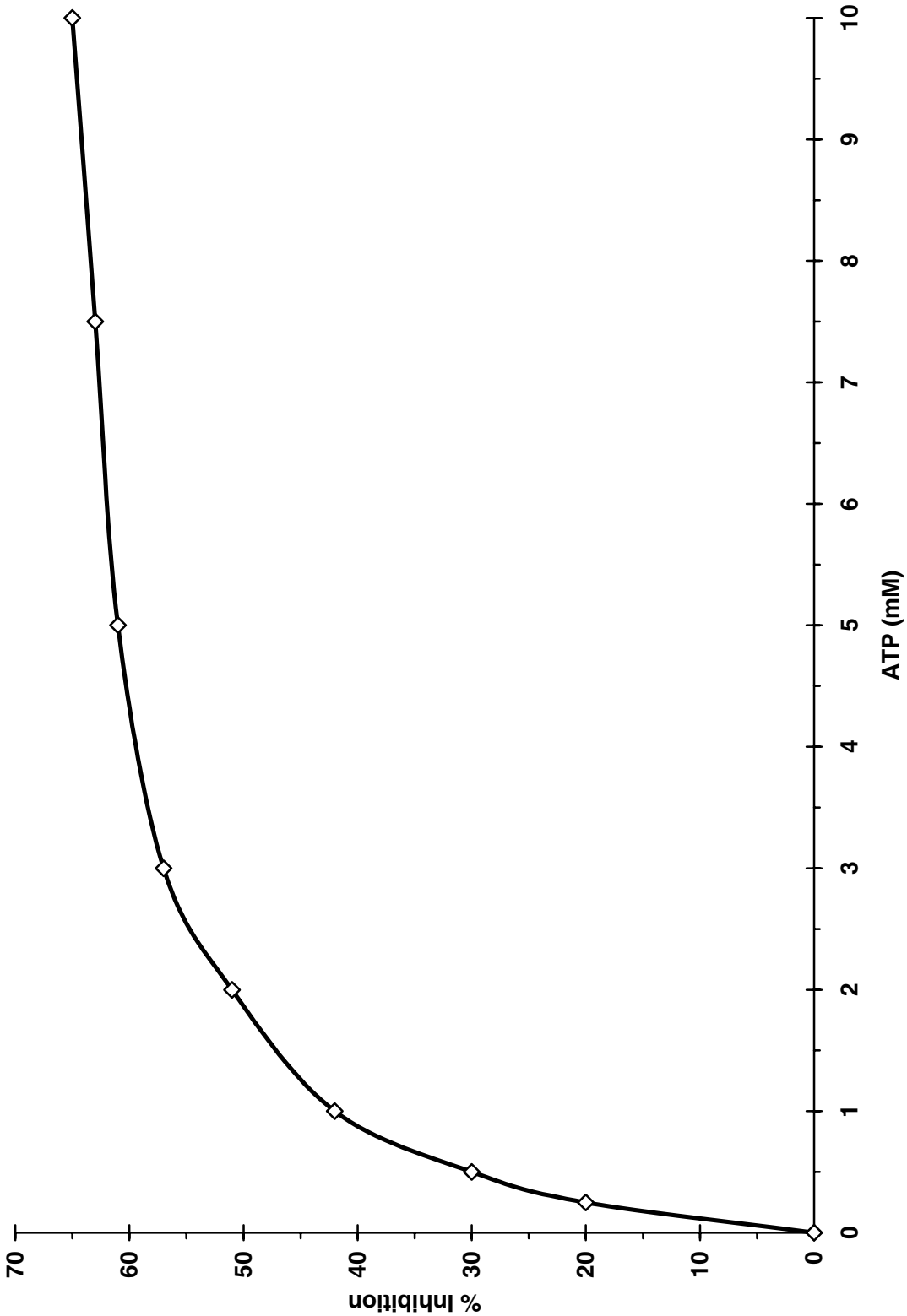

% Inhibition
0
10
20
30
40
50
60
70
ATP (mM)
0
1
2
3
4
5
6
7
8
9
10

Figure 9; Non-competitive Inhibition of Purified Glo-II-s Activity by ATP

The assay method is as described in Methods section. Velocity is in terms of μmol / min. of GSH produced from SLG. SLG concentrations were 0.125, 0.167, 0.25, 0.5, and 0.75 mM. ATP concentrations were 0, 0.4, 0.6, and 0.8 mM. Lines were generated using a computerized linear regression program. The K_M of the enzyme for the substrate is 0.54 mM.

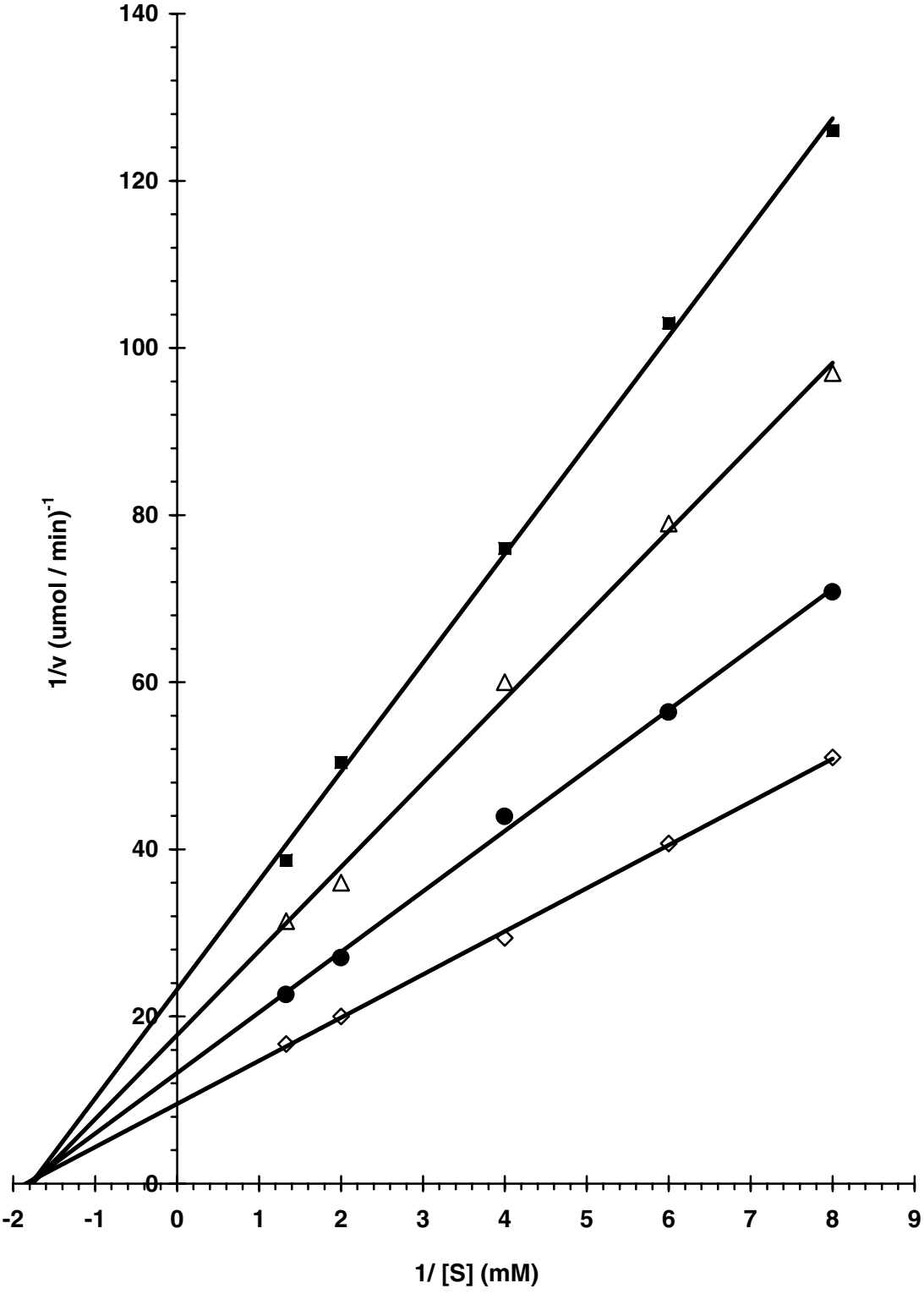

140
120
100
80
60
40
20
0
1/v (umol / min)-1
-2
-1
0
1
2
3
4
5
6
7
8
9
1/ [S] (mM)

Figure 10; Inhibition of Glo-II-s by ATP: Dixon Plot

The data from Figure 9 were used to construct the plot. Lines were generated using a computerized polynomial regression program.

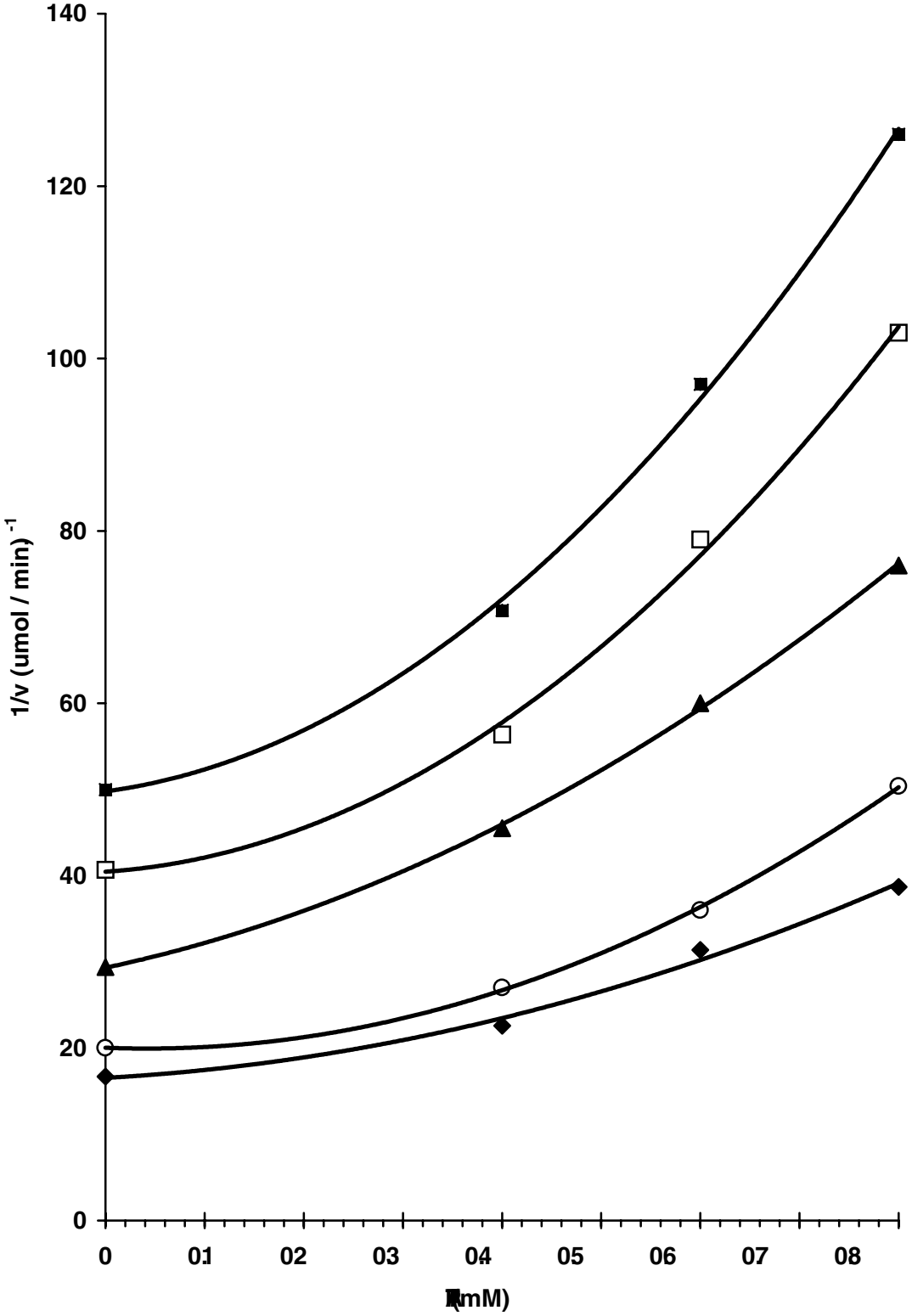

140
120
100
80
60
40
20
0
1/v (umol / min) -1
0
01
02
03
04
05
06
07
08
(mM)

Figure 11; Inhibition of Glo-II-s by ATP: Slope versus Inhibitor Concentration Plot

The data from Figure 9 were used to construct the plot. Lines were generated using a computerized polynomial regression program.

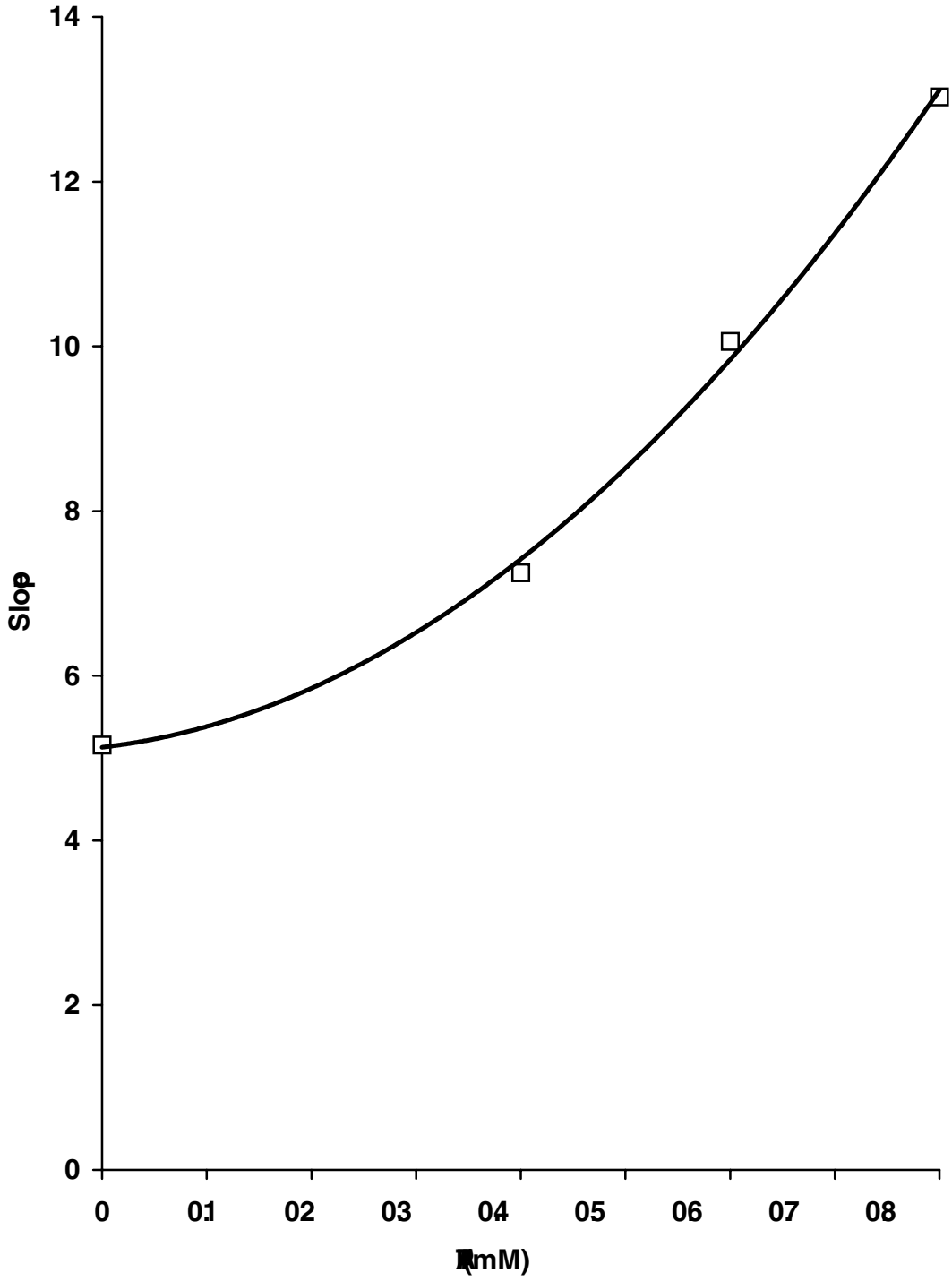

14
12
10
8
6
4
2
0
Slope
0
01
02
03
04
05
06
07
08
I (mM)

Figure 12; Glo-II-s, Eadie Schatchard Plot

Assays were conducted as described in the Methods section. Substrate concentrations used ranged from 0.025 mM to 0.80 mM. Lines were generated using a computerized polynomial regression program. Enzyme velocity (v) is in μmol / min.

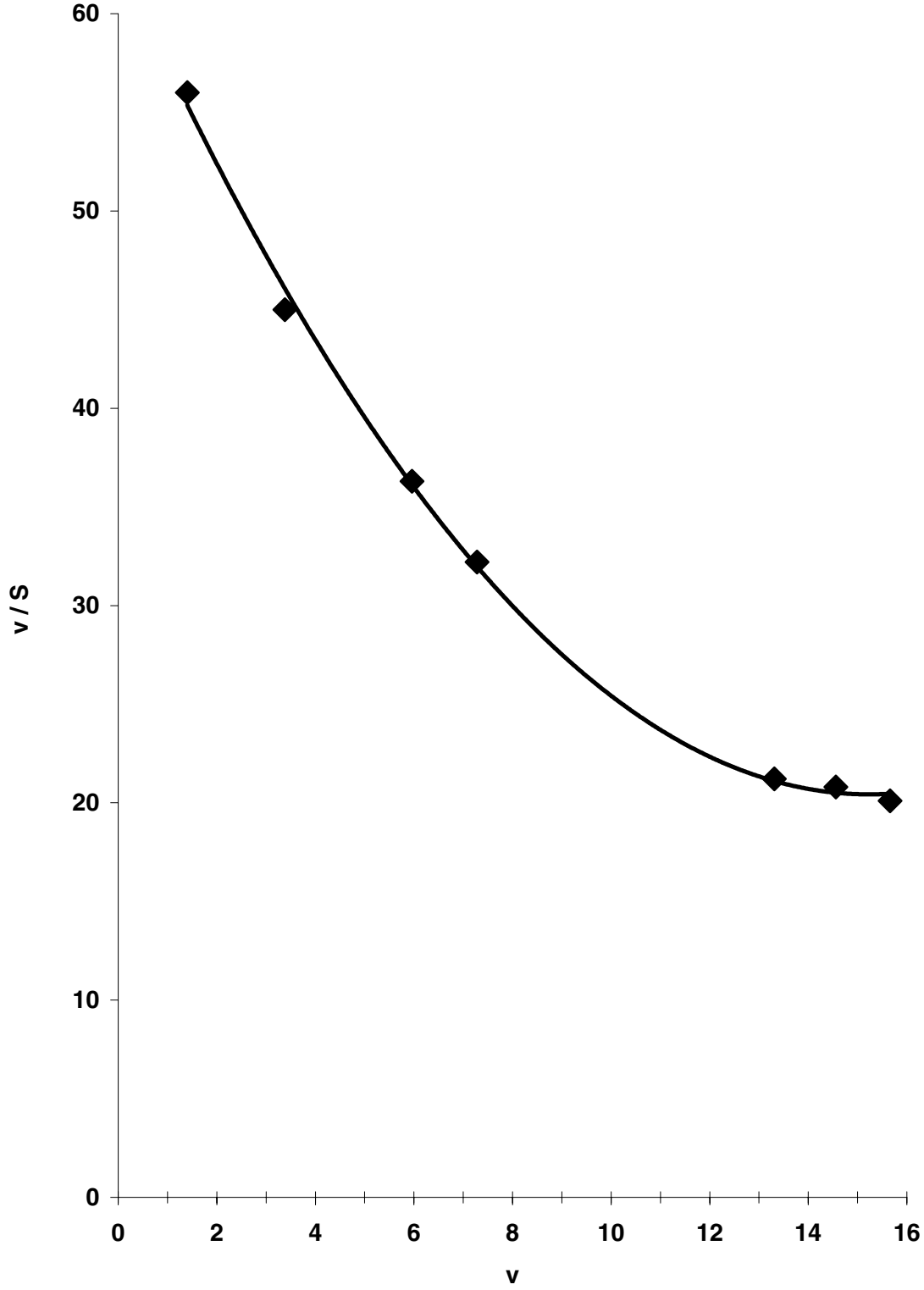

60
50
40
30
20
10
0
v / S
0
2
4
6
8
10
12
14
16
v

Figure 13; Desensitization of Glo-II-s by Incubation at 12^0C

Glo-II-s (2.5 U / ml) was incubated at 12^0C and aliquots were removed, diluted, and assayed periodically for activity. Assays were conducted in the absence of ATP (uninhibited activity) and in the presence of 2 mM ATP (inhibited activity) as described in the Methods section. SLG concentration in the assay was 0.25 mM. Data are plotted as % activity or % inhibition versus time.

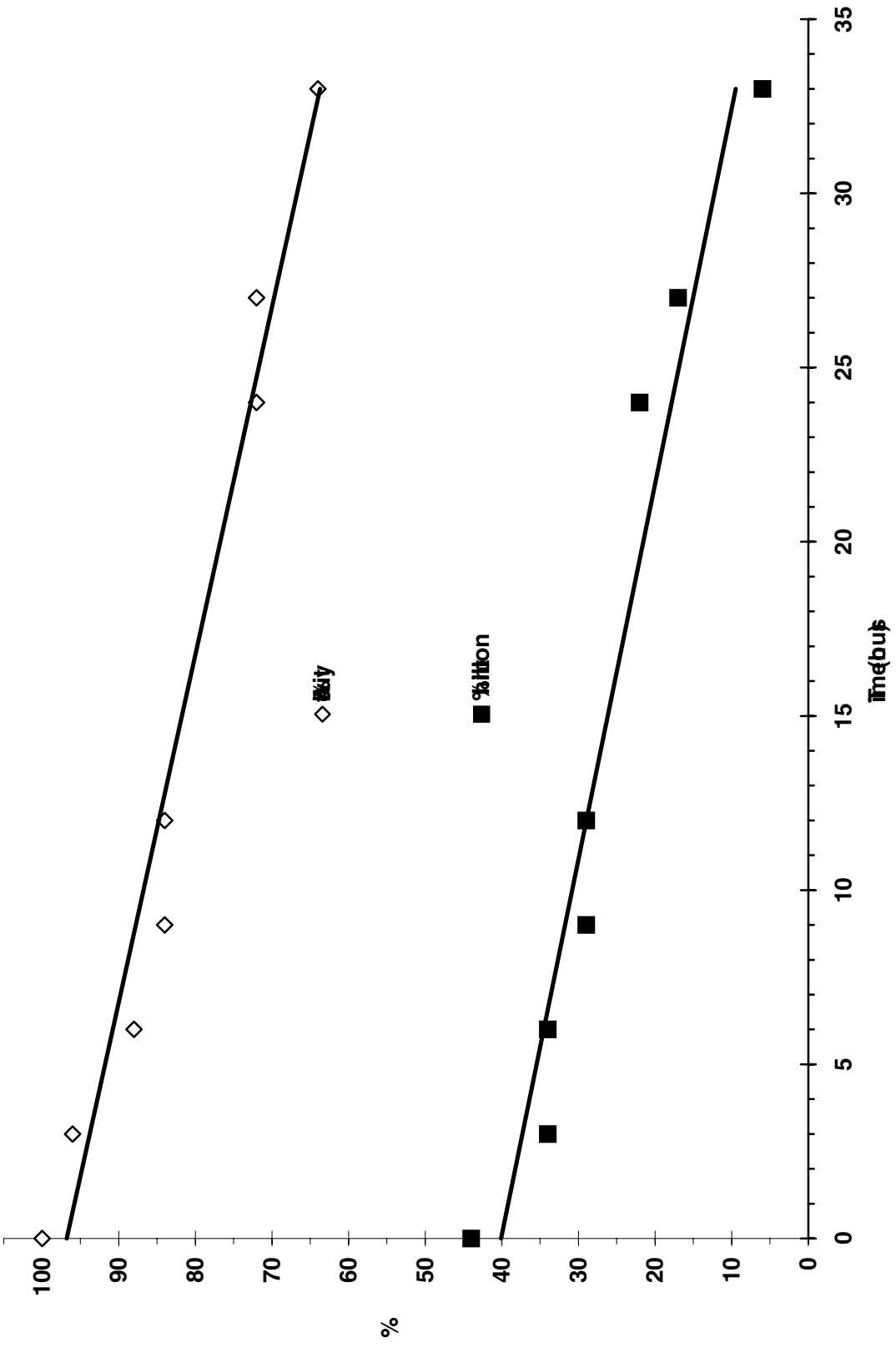

100
90
80
70
60
50
40
30
20
10
0
%
0
5
10
15
20
25
30
35

Figure 14; Desensitization of Glo-II-s by Incubation with ATP

Samples of Glo-II-s (20 U / ml) with or without 5 mM ATP, at designated times, were withdrawn and diluted into the assay medium such that residual nucleotide concentration from the incubation was 6 μM , a non-inhibitory concentration. Assays were conducted at 0.25 mM SLG to determine any change in the hydrolase activity of the incubated enzyme. Assays were also conducted at 0.25 mM SLG in the presence of 2 mM ATP to determine any change in inhibition properties. The control underwent no change in hydrolase activity or % inhibition during the incubation (data not shown). Percent activity was determined by the ratio of the activity of the sample incubated with ATP to that of the control, where the activity of the control was designated at 100 %. The percent inhibition was calculated from the ratio of the inhibited activity to the uninhibited activity.

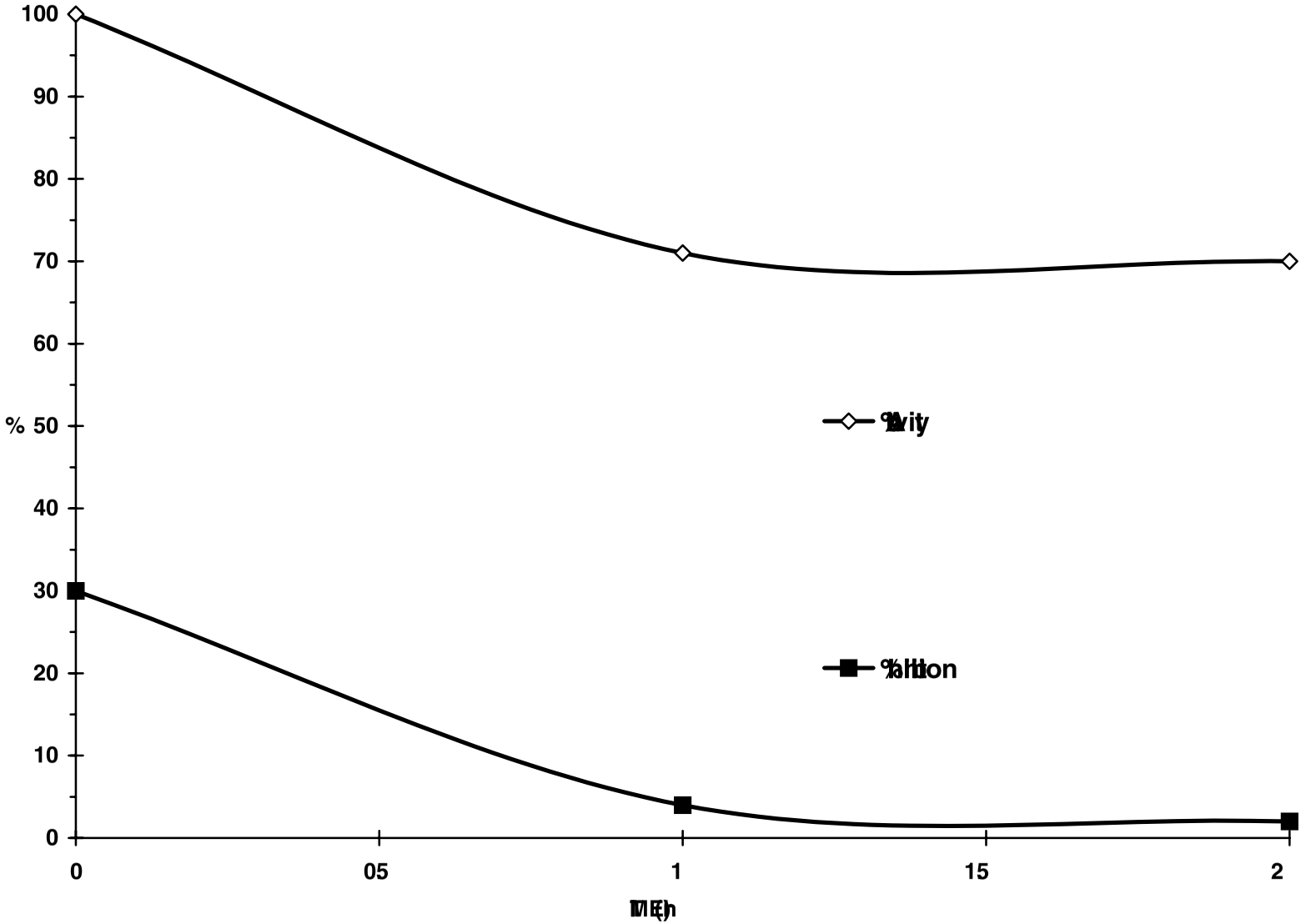

100
90
80
70
60
% 50
40
30
20
10
0
0
05
1
15
2

Figure 15; Percent Inhibition Profile of Purified Glo-II Activity by ATP, Glo-II-s vs. Glo-II-i Desensitized by Incubation at 12°C

Samples of Glo-II-s were assayed at varying levels of ATP (0 to 10 mM). All assays were conducted at 0.25 mM SLG. The percent activity was determined by the ratio of the activity of the enzyme in the presence of ATP to the activity of the enzyme in the absence of ATP. Samples of Glo-II-I, desensitized by incubation at 12°C, were then treated in the same manner for comparison.

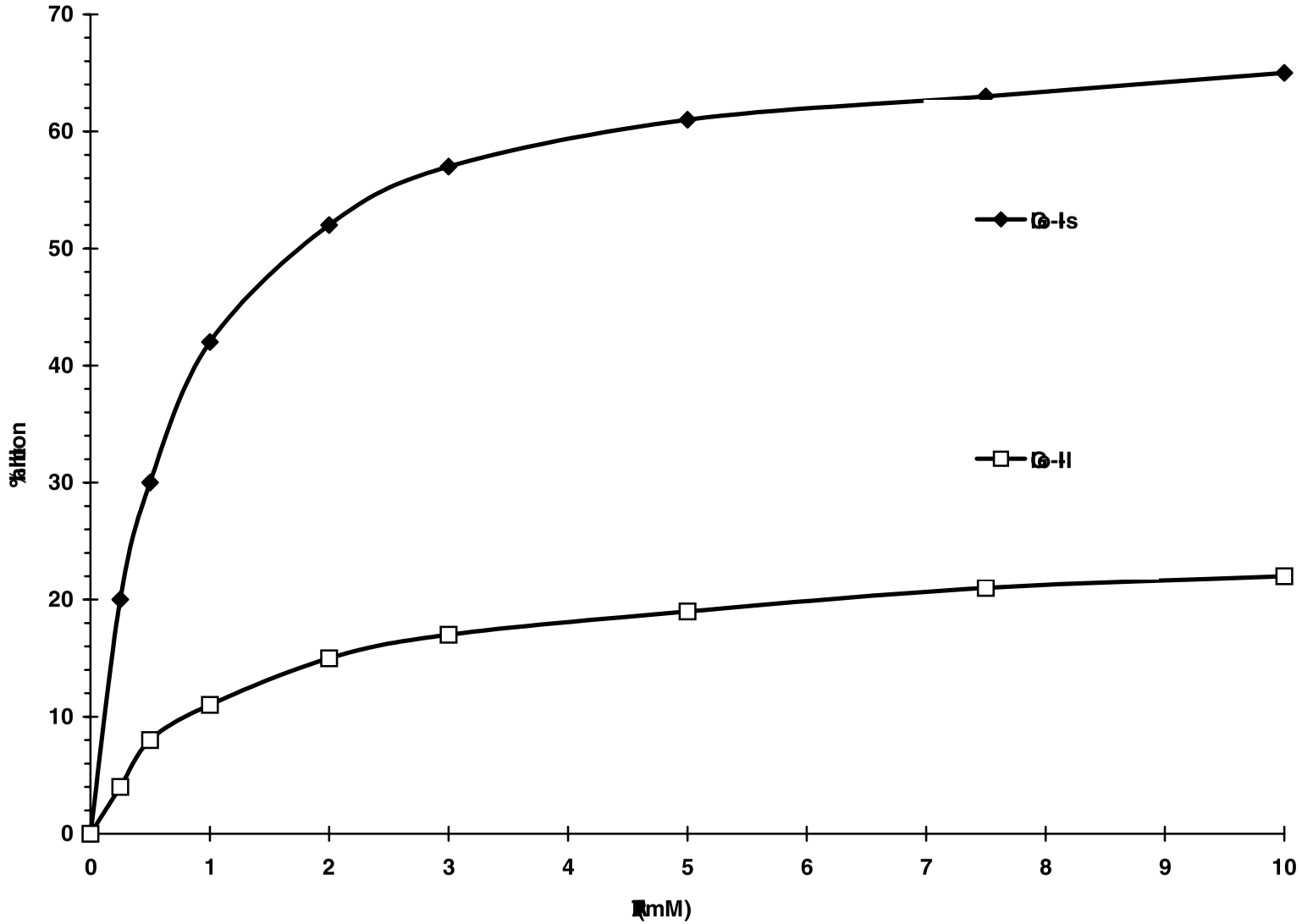

70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
(mM)

Figure 16; Lineweaver-Burk Plot of Glo-II-i Activity Desensitized by Incubation at 12°C

The assay method is as described in the Methods section. Velocity is in terms of μmol / min. of GSH produced from SLG. SLG concentrations were between 0.125 and 1 mM. Lines were generated using a computerized linear regression program. The K_M of the enzyme for the substrate is 0.22 mM.

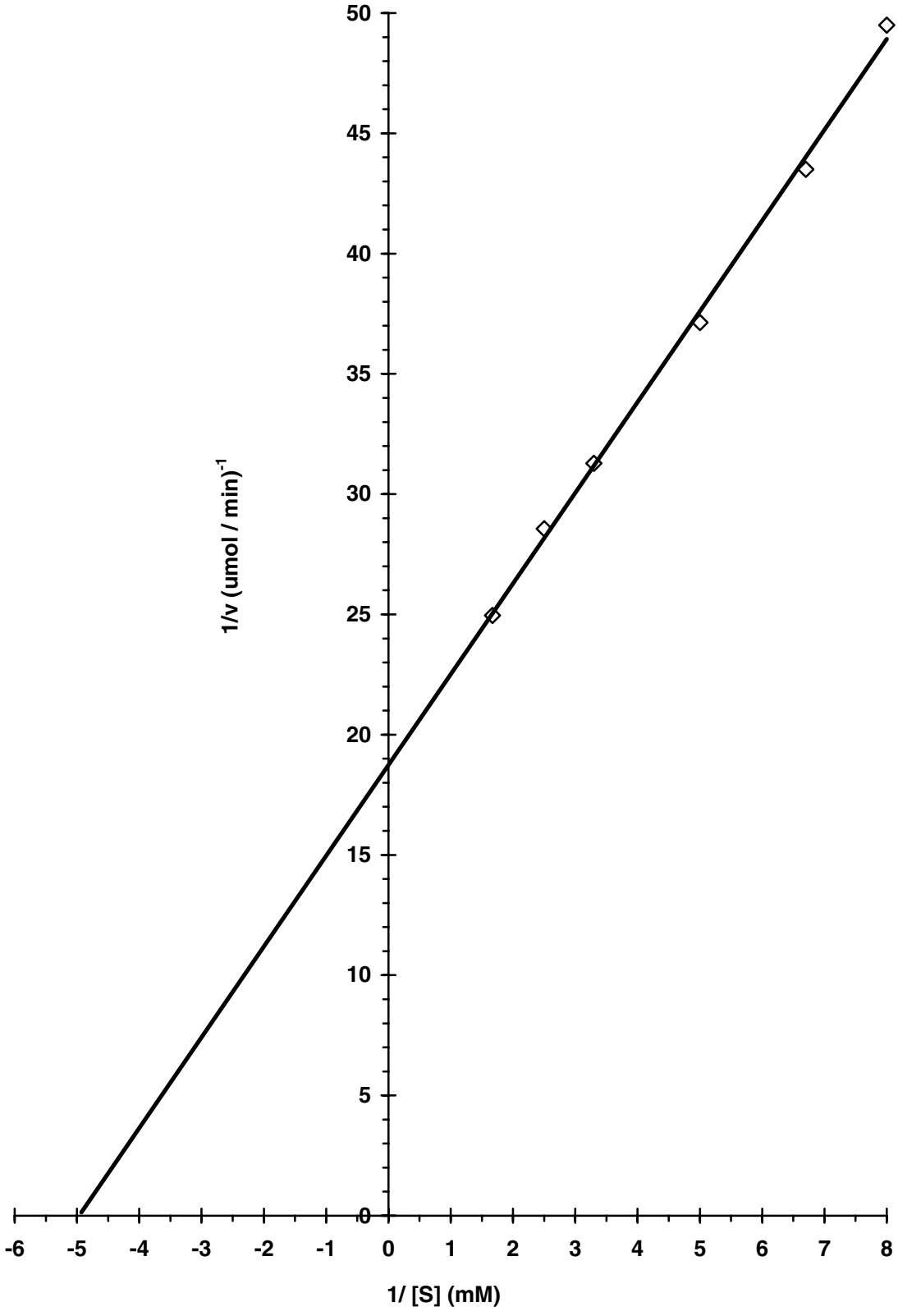

50
45
40
35
30
25
20
15
10
5
0
1/v (umol / min)-1
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
1/ [S] (mM)

Figure 17; Eadie – Scatchard Plot of Glo-II-i Activity Desensitized by Incubation at 12°C

The data from Figure 16 were used to construct the plot. The line was generated using a computerized linear regression program.

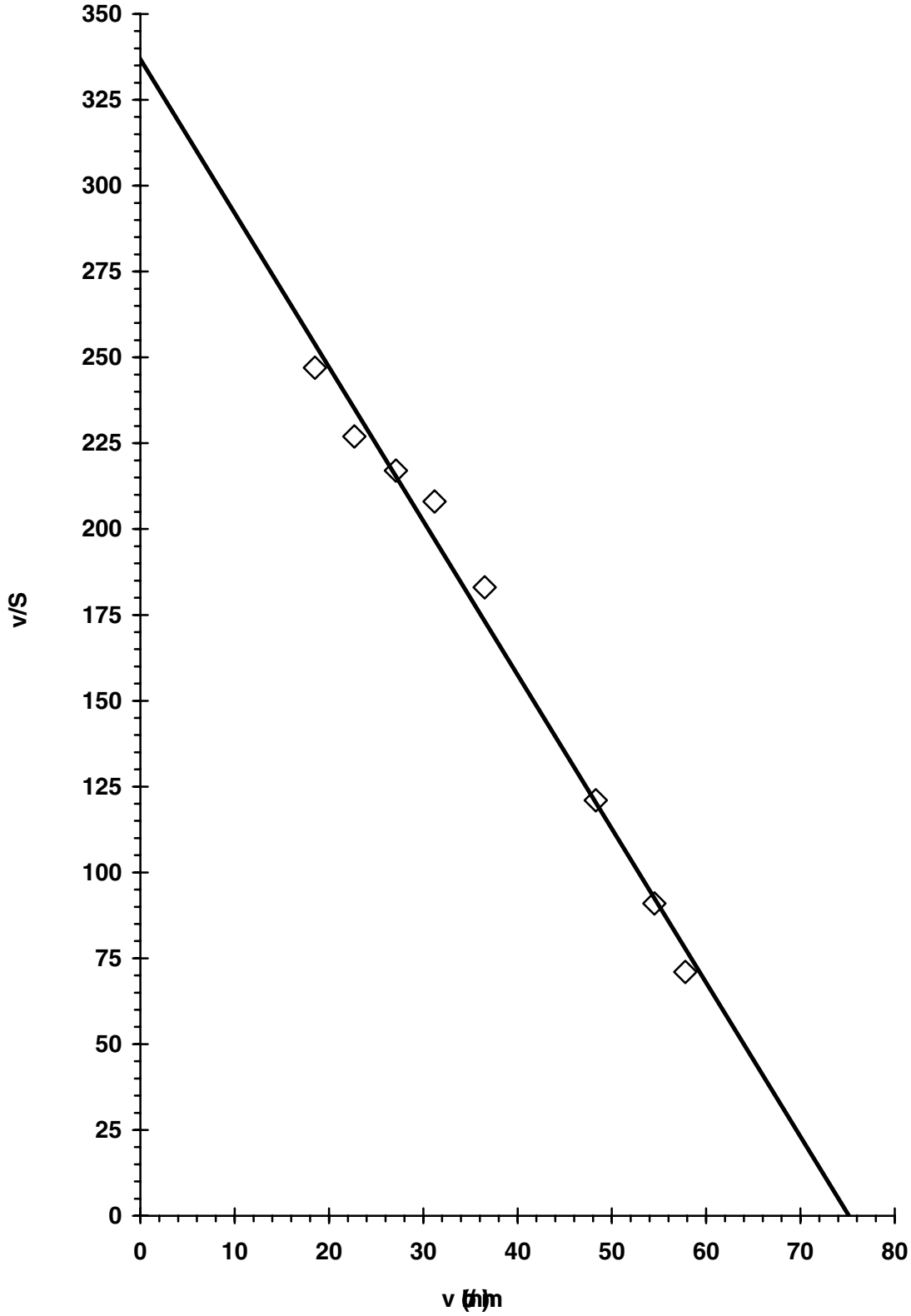

350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
v/S
0
10
20
30
40
50
60
70
80
v [m/s]

Figure 18; Percent Inhibition of Glo-II from Calf Liver by ATP, Glo-II-s vrs. Glo-II-i Desensitized by Incubation with ATP

Samples of Glo-II-s were assayed at varying levels of ATP (0 to 10 mM). All assays were conducted at 0.25 mM SLG. The percent activity was determined by the ratio of the activity of the enzyme in the presence of ATP to the activity of the enzyme in the absence of ATP. Samples of Glo-II-i (prepared by incubation with ATP) were then treated in the same manner.

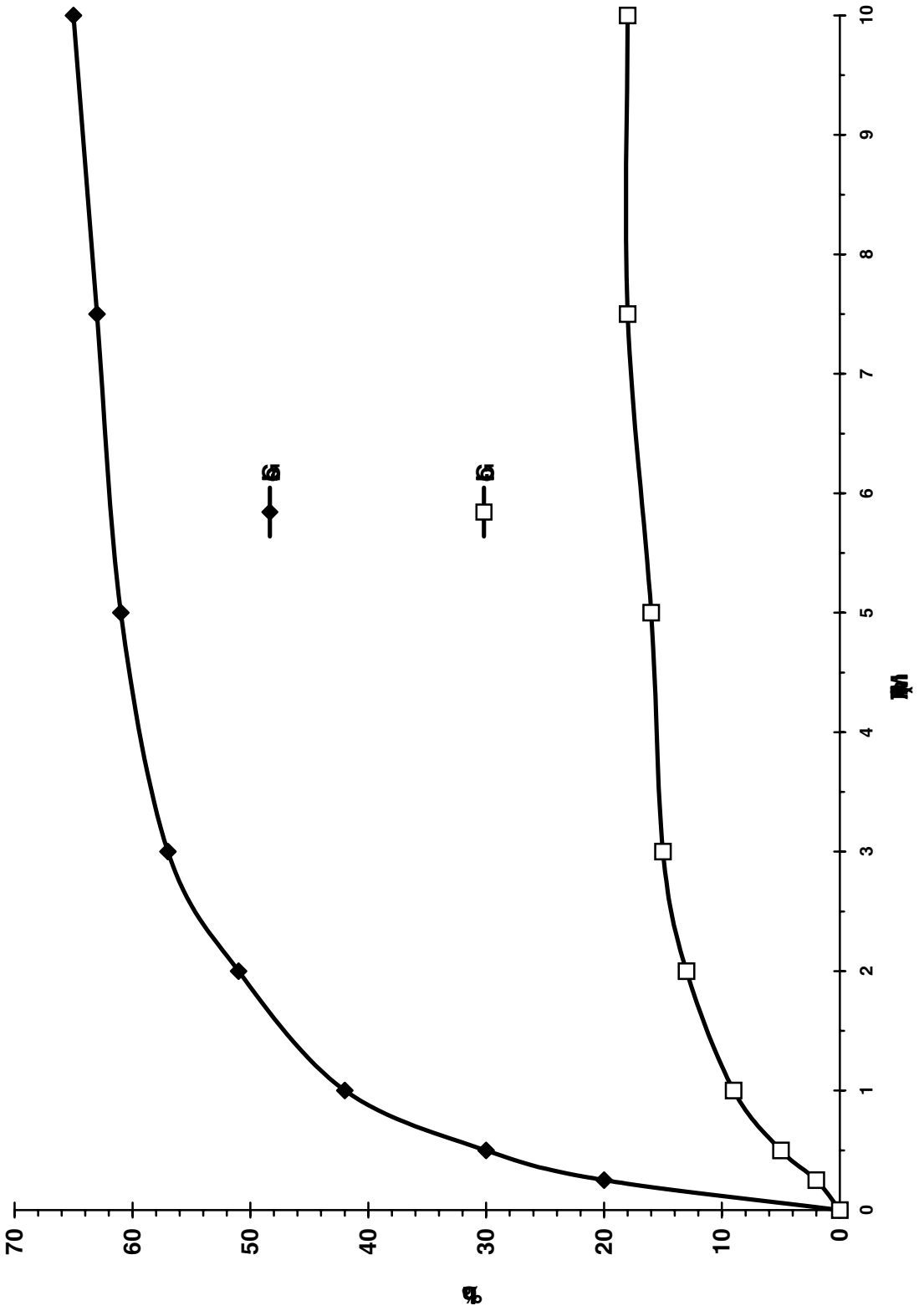

70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10

Figure 19; Lineweaver-Burk Plot of Glo-II-i Desensitized by Incubation with ATP

The assay method is as described in the Methods section. Velocity is in terms of μmol / min. of GSH produced from SLG. SLG concentrations were between 0.125 and 1 mM SLG. Lines were generated using a computerized linear regression program. The K_M of the enzyme for the substrate is 0.22 mM.

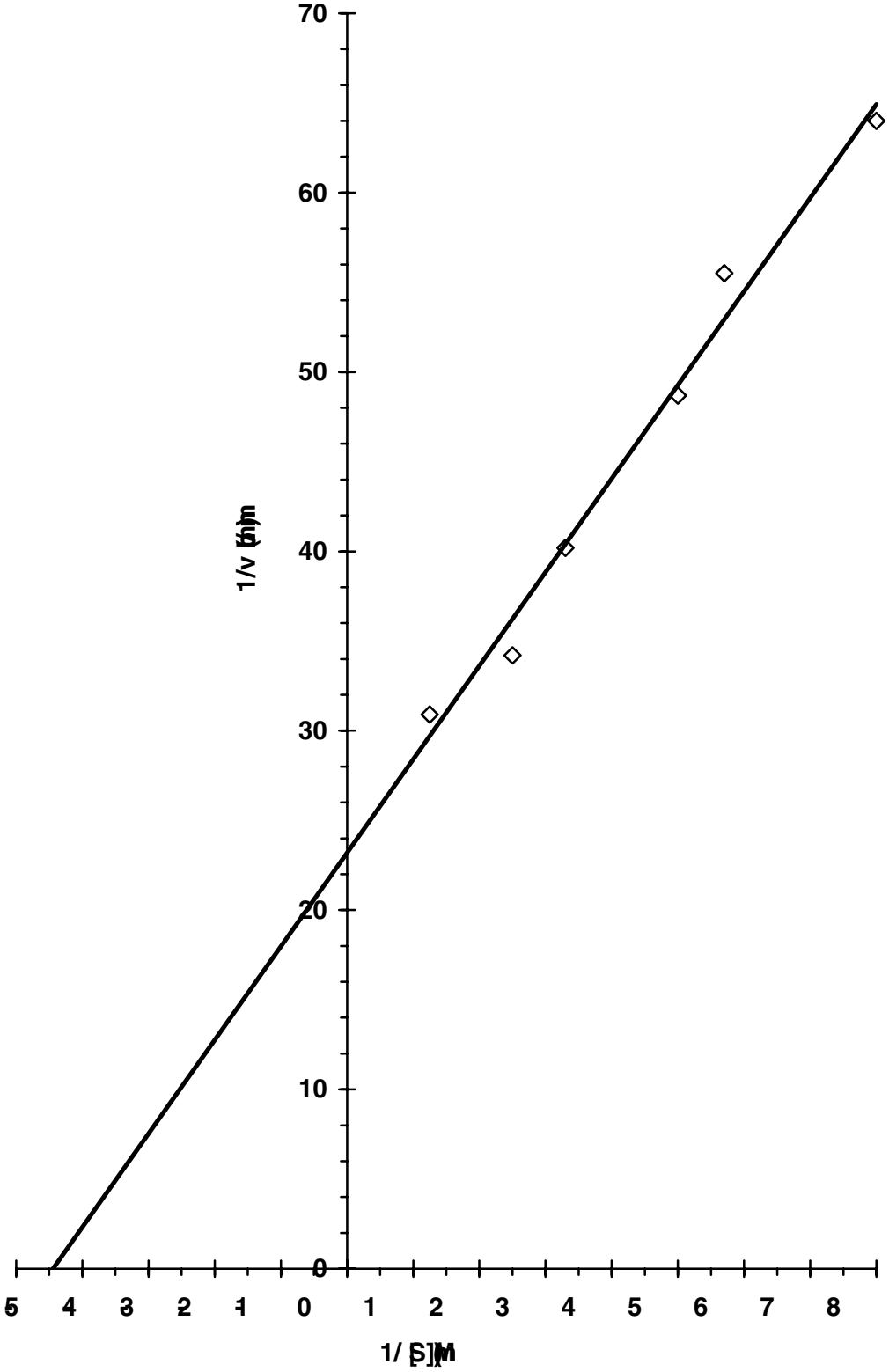

70
60
50
40
30
20
10
0
1/v (min/mM)
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
1/[S] (1/mM)

Figure 20; Eadie – Scatchard Plot of Glo-II-i Desensitized by Incubation with ATP

The data from Figure 19 were used to construct the plot. Lines were generated using a computerized linear regression program.

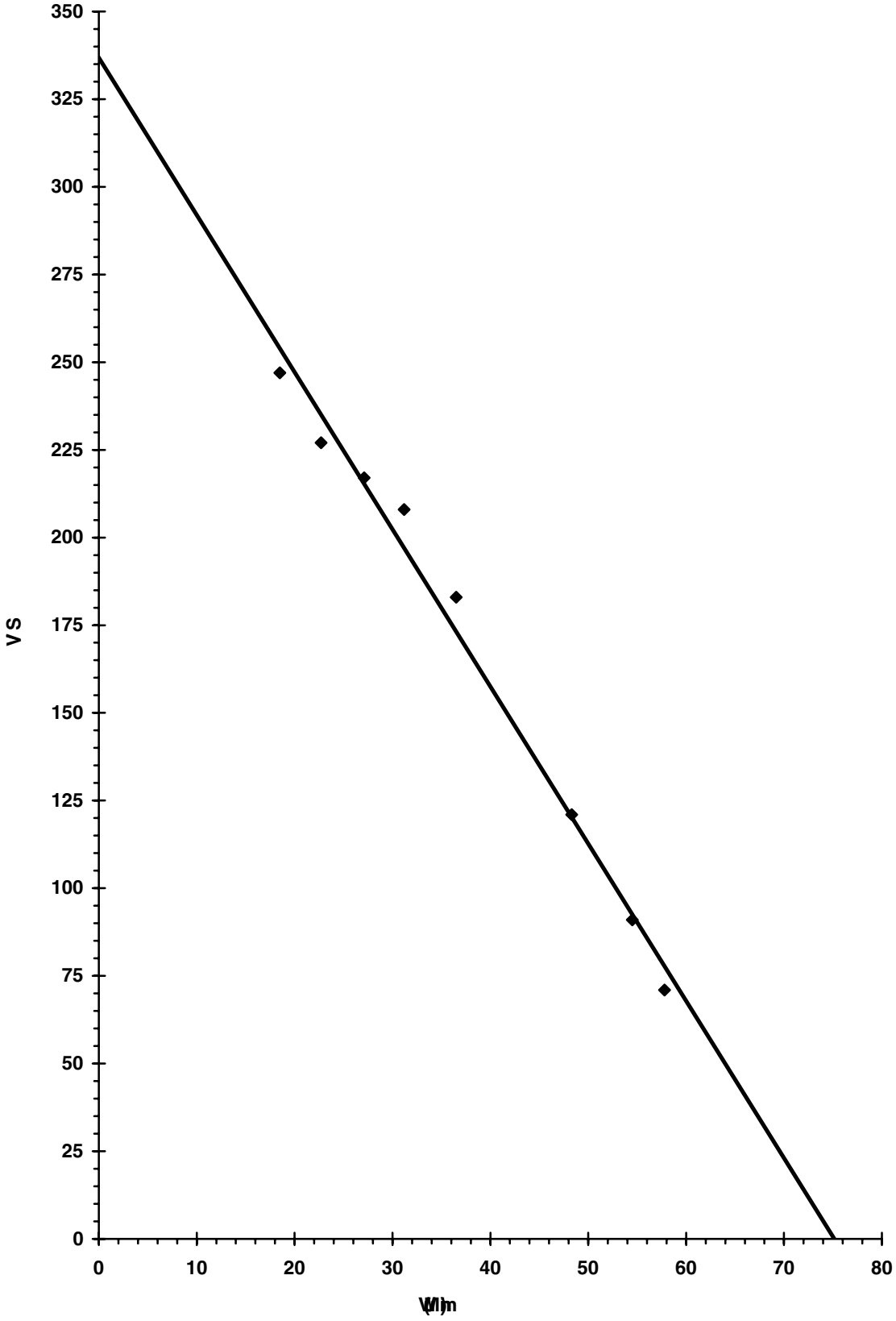

350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
VS
0
10
20
30
40
50
60
70
80

Figure 21; Comparison of the Vmax and K_M values of Glo-II-s and Glo-II-i

The Glo-II-s enzyme was de-sensitized by incubation with ATP (5 mM) as previously described. After re-purification of the sample, the resulting Glo-II-i preparation was compared to an equal protein concentration of the original Glo-II-s enzyme by the double reciprocal plot method of Lineweaver-Burk. The Glo-II-i form of the enzyme exhibited the characteristic reduction in K_M value (from 0.54 mM to 0.22 mM) that had been described previously. No significant change in the value for Vmax was observed.

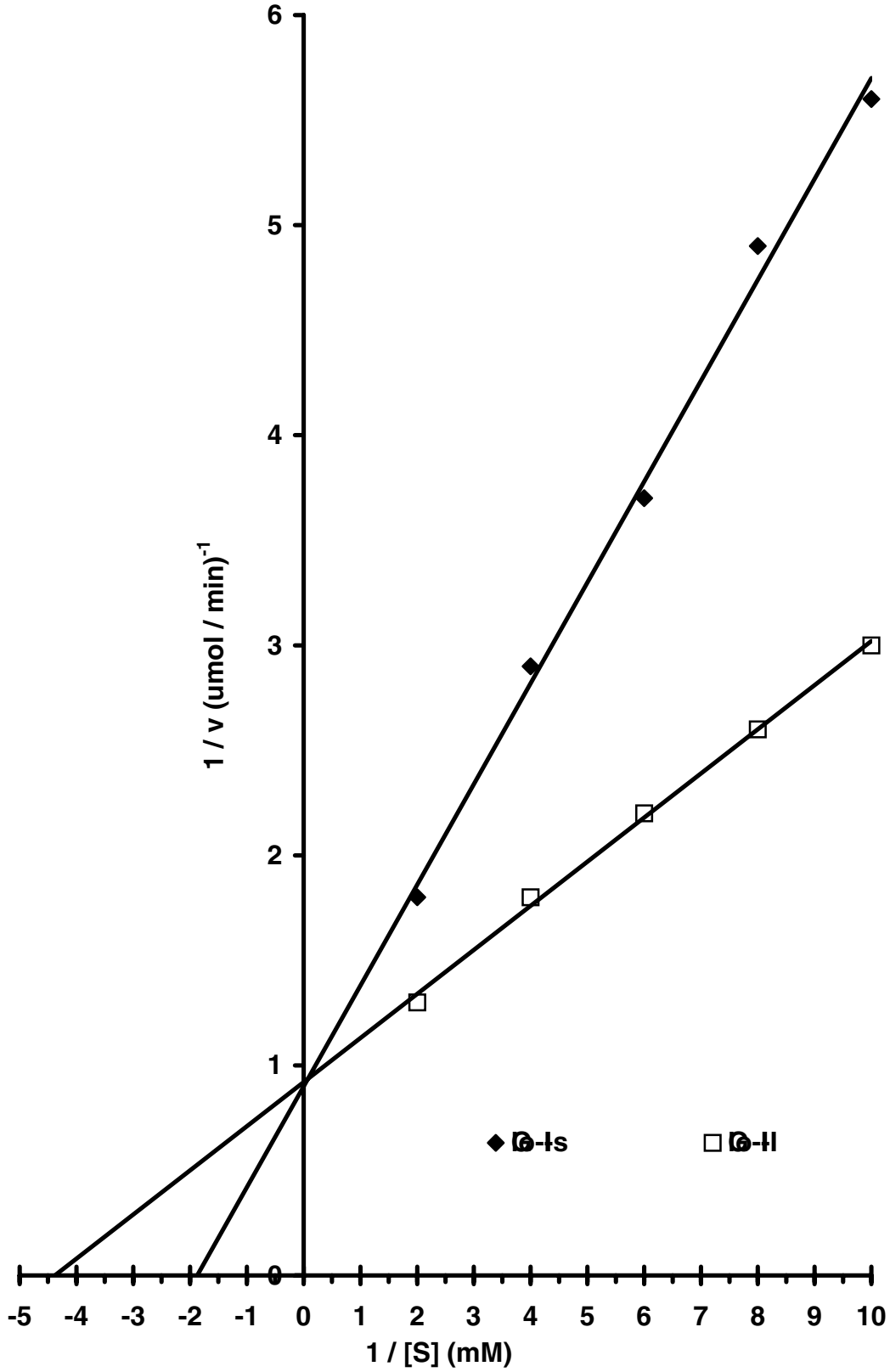
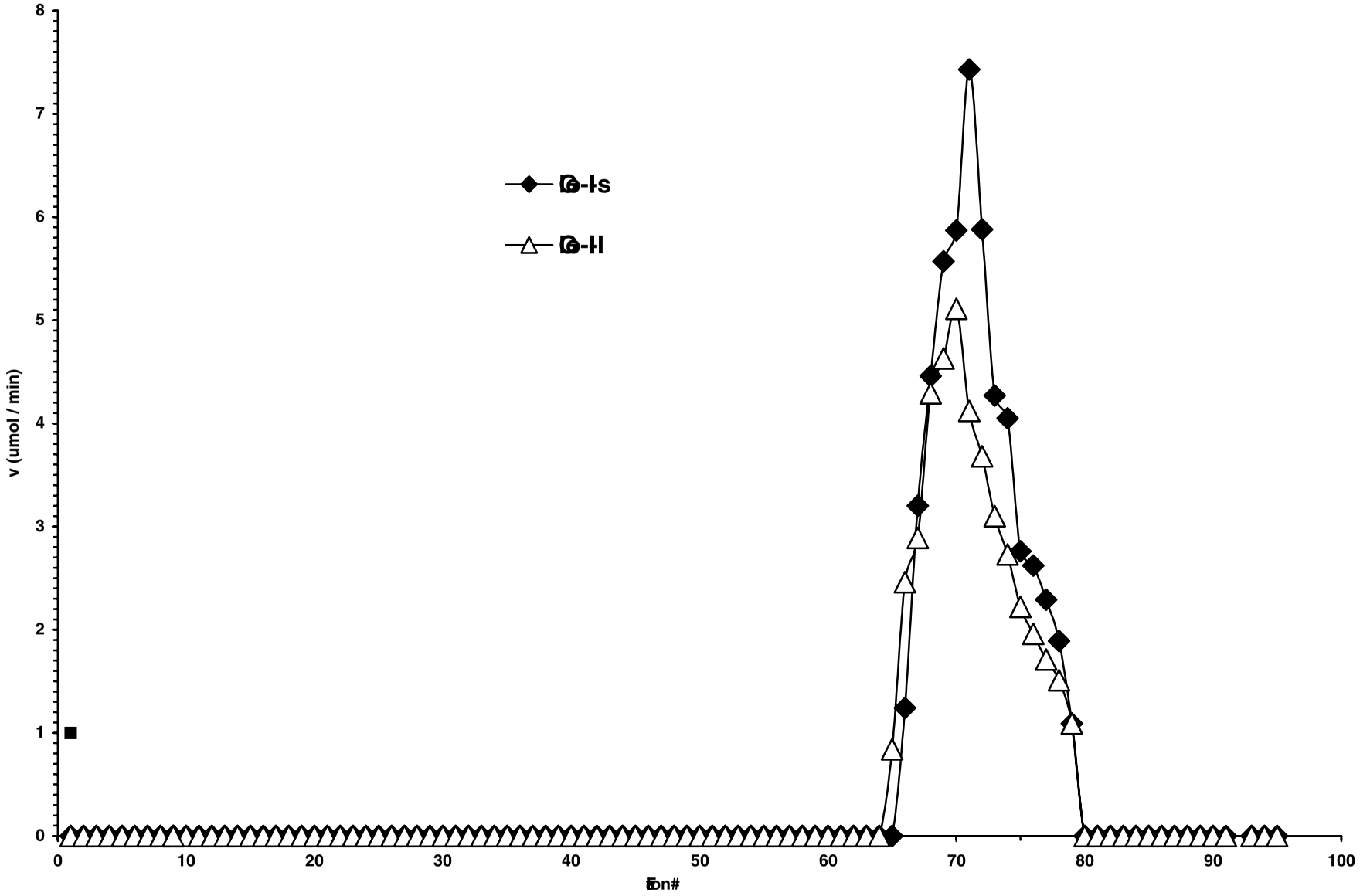

6
5
4
3
2
1
0
1 / v (umol / min)-1
-5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
1 / [S] (mM)

Figure 22; Comparison of FPLC Elution Profiles of Glo-II-s And Glo-II-i Desensitized by Incubation at 12°C

The elution profiles of Glo-II samples were determined on a Superdex 200 HR 10/30 high performance gel filtration column. The column was first equilibrated with a buffer medium containing 40 mM TES, 100 mM NaCl, at pH 7.2. Samples containing 50 ug of purified Glo-II, 100 uL volume, in buffer A were then applied to the column. Protein was eluted from the column at a flow rate of 0.5 mL / min. Fractions (0.25 mL / fraction) were collected and assayed as described previously.



[61]

Figure 23; Gel Filtration Profiles of Glo-II-s - Effect of the presence of ATP

Concentrated Glo-II-s (50 µl of a 20 U / ml solution) was applied to a G-75 Sephadex column (50 cm × 1 cm), equilibrated with buffer A containing 100 mM NaCl. Fractions (0.25 ml) were eluted from the column with the same buffer at a flow rate of 0.25 ml / min. and assayed as previously described. The column was then washed extensively with buffer A containing 100 mM NaCl and 1 mM ATP. Concentrated Glo-II-s (50 µl of a 20 U / ml solution) was applied to the column and eluted with the buffer A containing 100 mM NaCl and 1 mM ATP. Fractions were collected and assayed as described above.

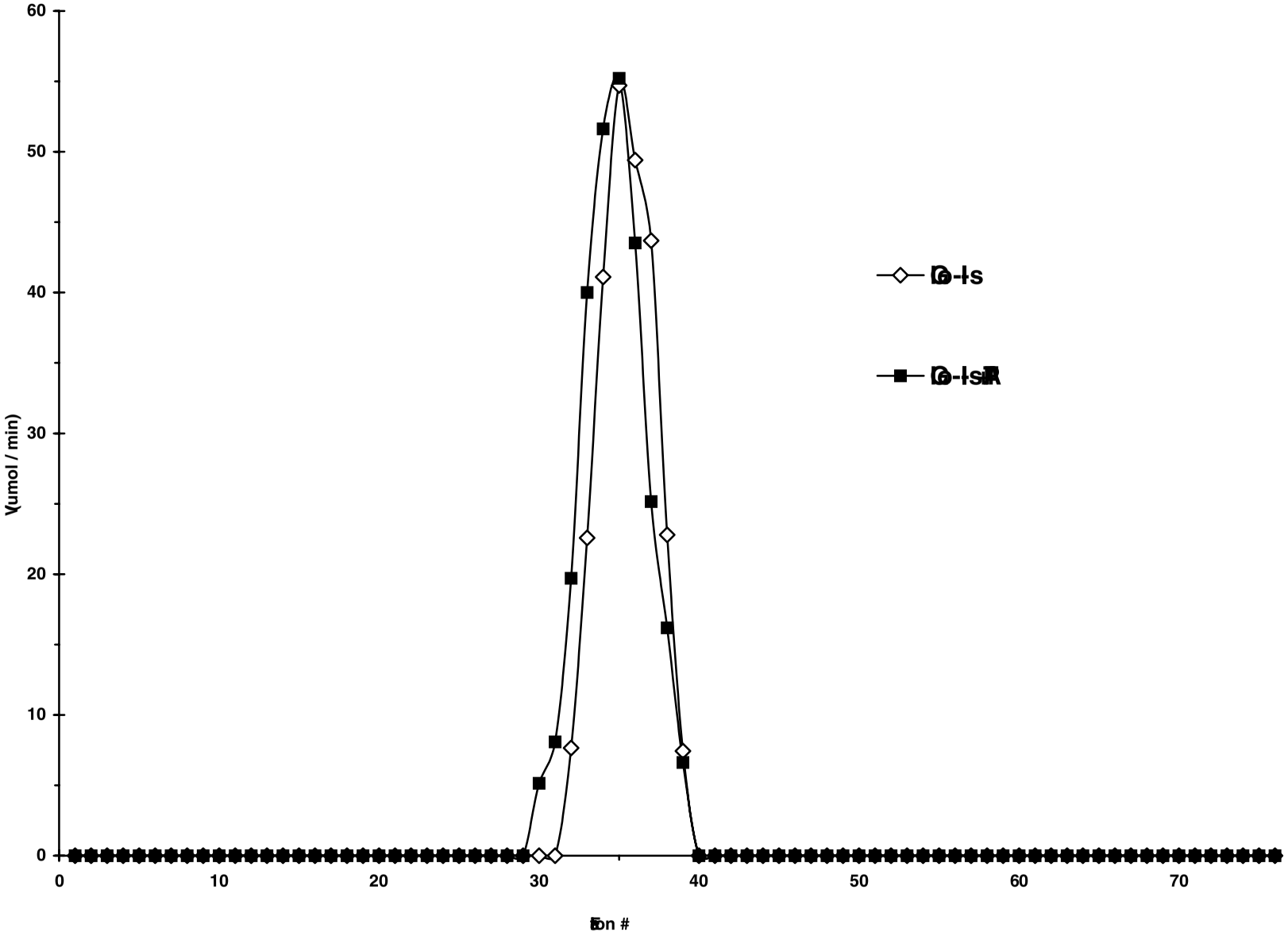

60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Fraction #
V (umol / min)

Figure 24; Comparison of the Circular Dichroism Spectra of Glo-II-s and Glo-II-i Desensitized by Incubation at 12°C

Purified samples of Glo-II were analyzed by circular dichroic analysis over the far-ultraviolet CD spectra, range 190 – 260 nm, 0.1 cm path length, constant bandwidth of 1.50 nm, at 15°C with a Jasco spectropolarimeter. Spectra were analyzed using 3 seconds of integration time per reading, and were repeated 3 times. Signals at each wavelength were then averaged and digitally filtered by an AVIV 60DS version 4.1g digital-smoothing program. The process was repeated for corresponding blanks and spectra were corrected accordingly. The concentration of protein in the sample was 8 μM. The buffer used was 10 mM TRIS, pH 7.4.

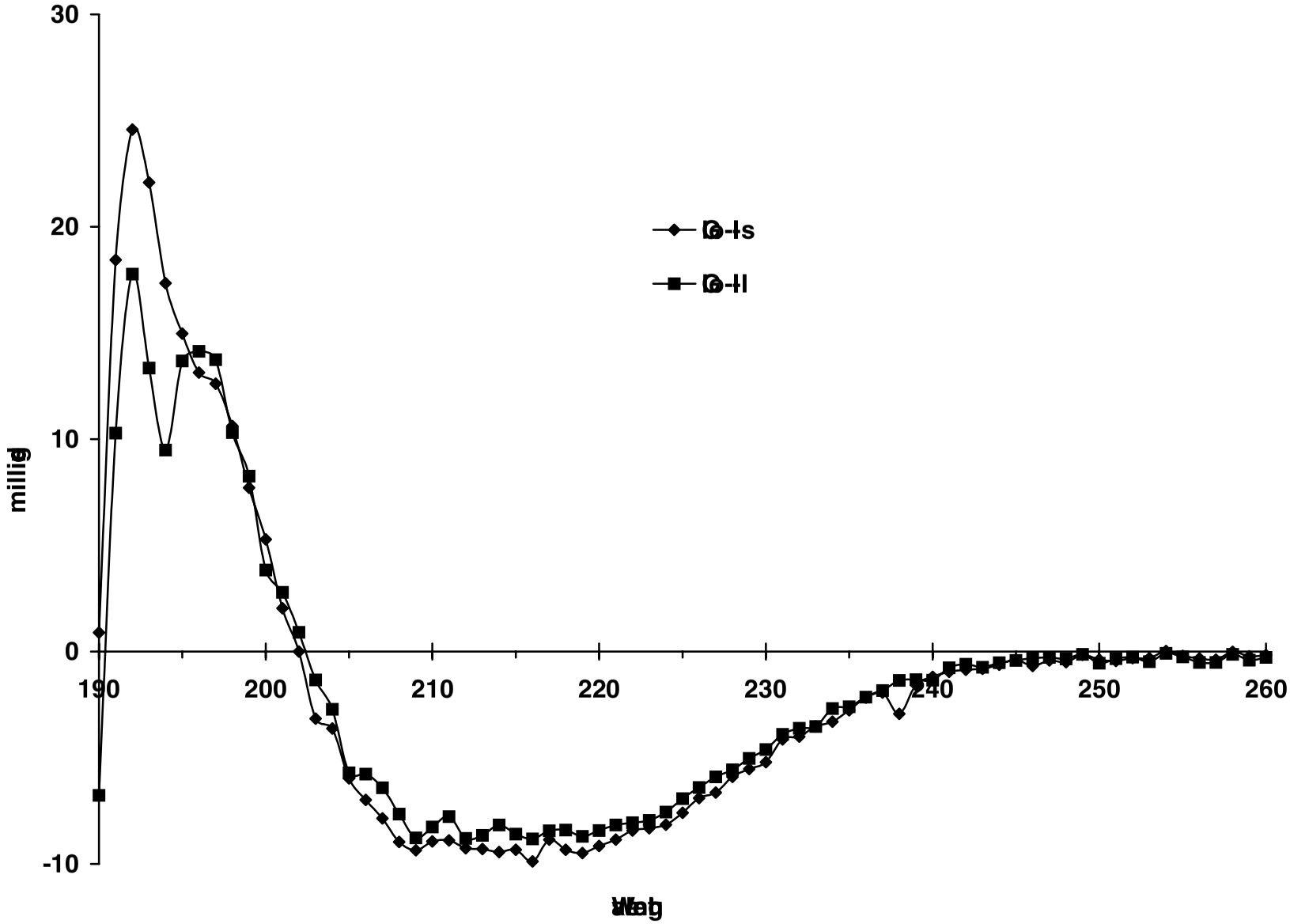

30
20
10
0
-10
190
200
210
220
230
240
250
260

Figure 25; Temperature Denaturation of Profiles of Glo-II-sversus Glo-II-i Desensitized by Incubation at 12°C

Purified samples of Glo-II were denatured and analyzed by circular dichroic analysis at 220 nm, 0.1 cm path length, constant bandwidth of 1.50 nm, with a Jasco spectropolarimeter. Readings were analyzed using 3 seconds of integration time per reading, and were repeated 3 times. The range of temperatures in which data were collected was from 25°C to 89°C, with temperature steps of 2.0°C, 0.2 min. equilibration time. Signals at each temperature were then averaged and digitally filtered by an AVIV 60DS version 4.1g digital-smoothing program. The process was repeated for corresponding blanks and spectra were corrected accordingly. The concentration of protein in the sample was 8 μM. The buffer used was 10 mM TRIS, pH 7.4.

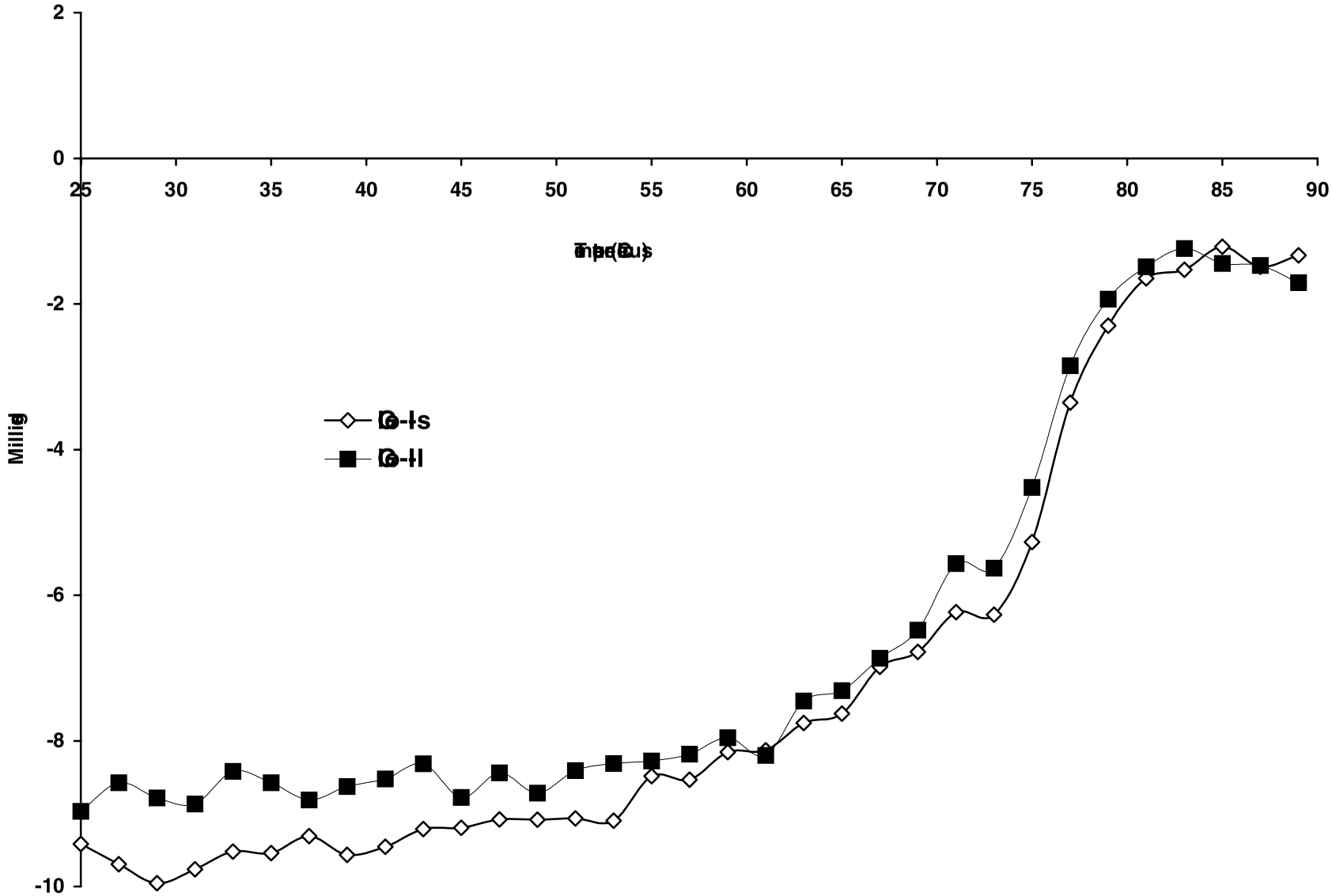

2
0
-2
-4
-6
-8
-10
25
30
35
40
45
50
55
60
65
70
75
80
85
90

Figure 26; Comparison of the Tryptophan Fluorescent Emission Spectra of Glo-II-s versus Glo-II-i Desensitized by Incubation at 12°C

The fluorescent emission spectra of the tryptophan residues of purified samples of Glo-II were analyzed in an SLM-Amico Bowman II luminescence spectrometer over a range of 310 to 500 nm. The protein concentration of all samples analyzed was 8 μM. Buffer used was 10 mM TRIS, pH 7.4.

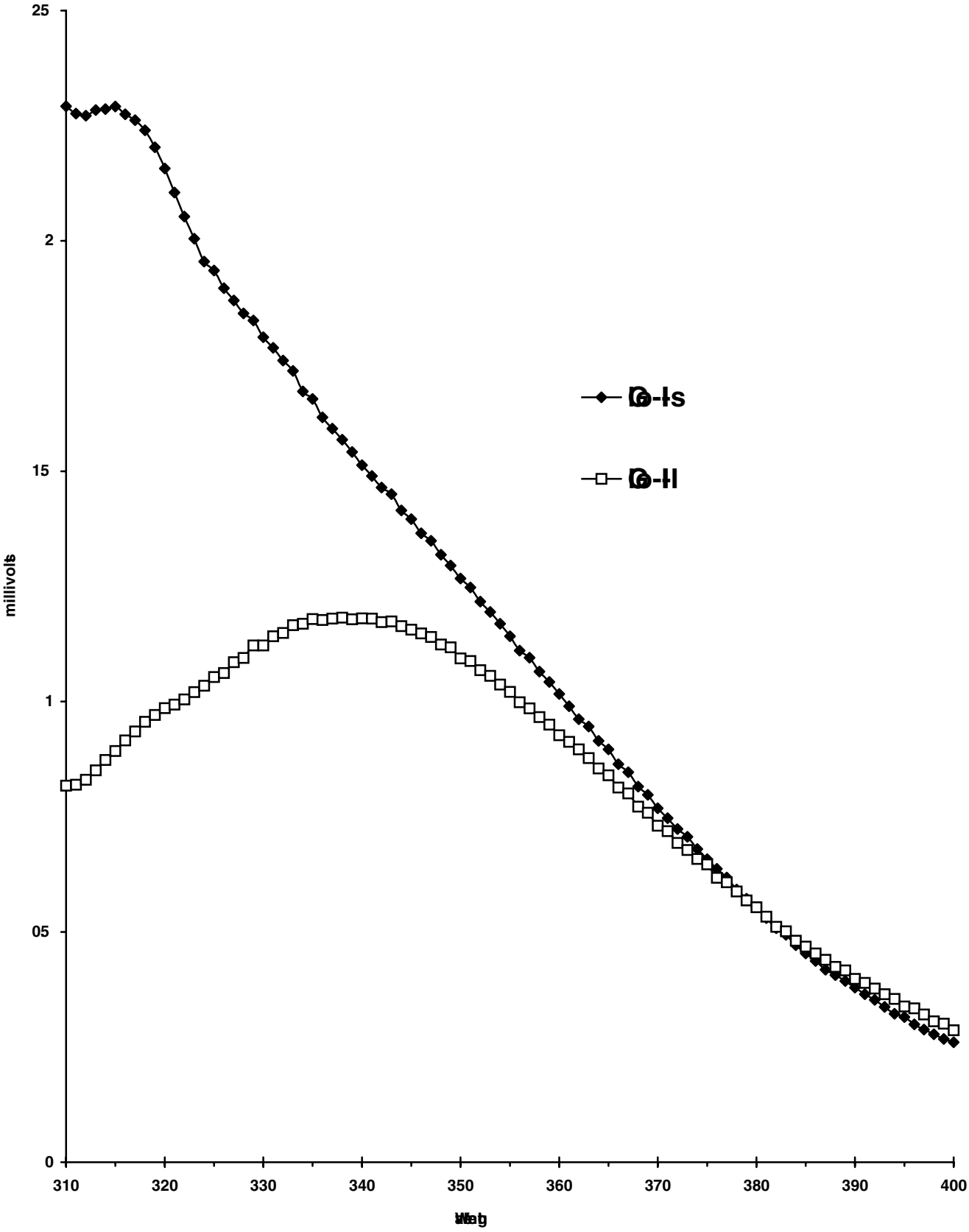

25
2
15
1
05
0
millivolts
310
320
330
340
350
360
370
380
390
400

Figure 27; Comparison of the Fluorescent Anisotrophy Spectra of Glo-II-s and Glo-II-i Desensitized by Incubation at 12°C

The fluorescent emission spectra of purified samples of Glo-II were determined in an SLM-Amico Bowman II luminescent spectrometer over a range of 310 to 350 nm. The excitation wavelength used was 280 nm. Analysis of the samples was conducted by anisotropic analysis of the spectra. The protein concentration of all samples analyzed was 8 μM. Buffer used was 10 mM TRIS, pH 7.4.

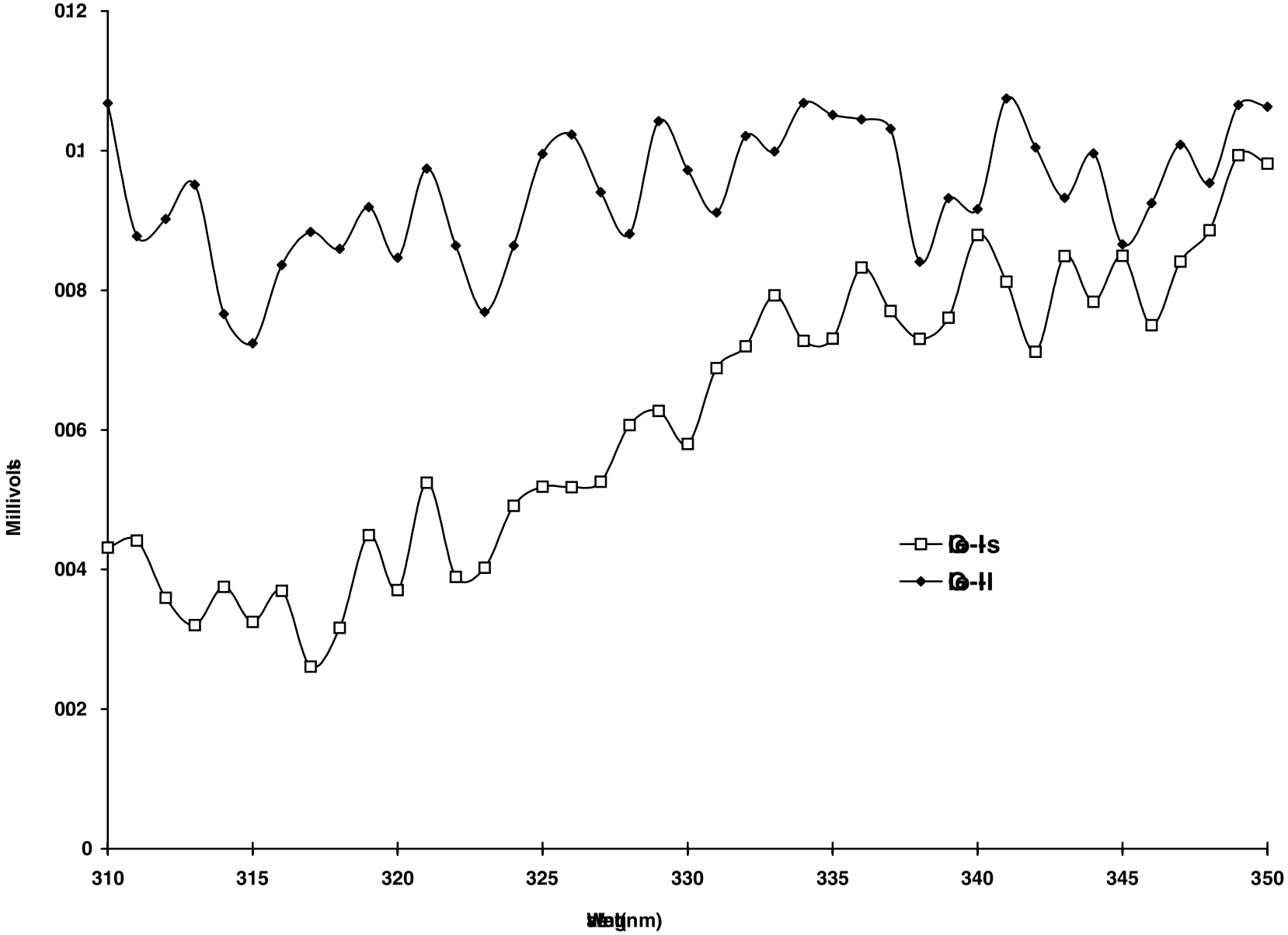

012
01
008
006
004
002
0
310
315
320
325
330
335
340
345
350
Millivolts
Wavelength (nm)

Figure 28; Effect of Histidine Modification by Diethylpyrocarbonate on Glo-II-s

Chemical modification of histidine residues was done at 1°C using 1-4 IU of Glo-II-s in 30 mM TRIS, pH 6.0. The modification reaction was started by the addition an aliquot of a DEPC / EtOH stock solution (1% EtOh, 6 mM DEPC final concentration). Aliquots were removed at appropriate time intervals and assays were conducted as previously described with and without 2 mM ATP. Results were recorded as percent residual activity or as percent residual inhibition. A control was treated similarly and used for comparison. These values were plotted against the time of incubation.

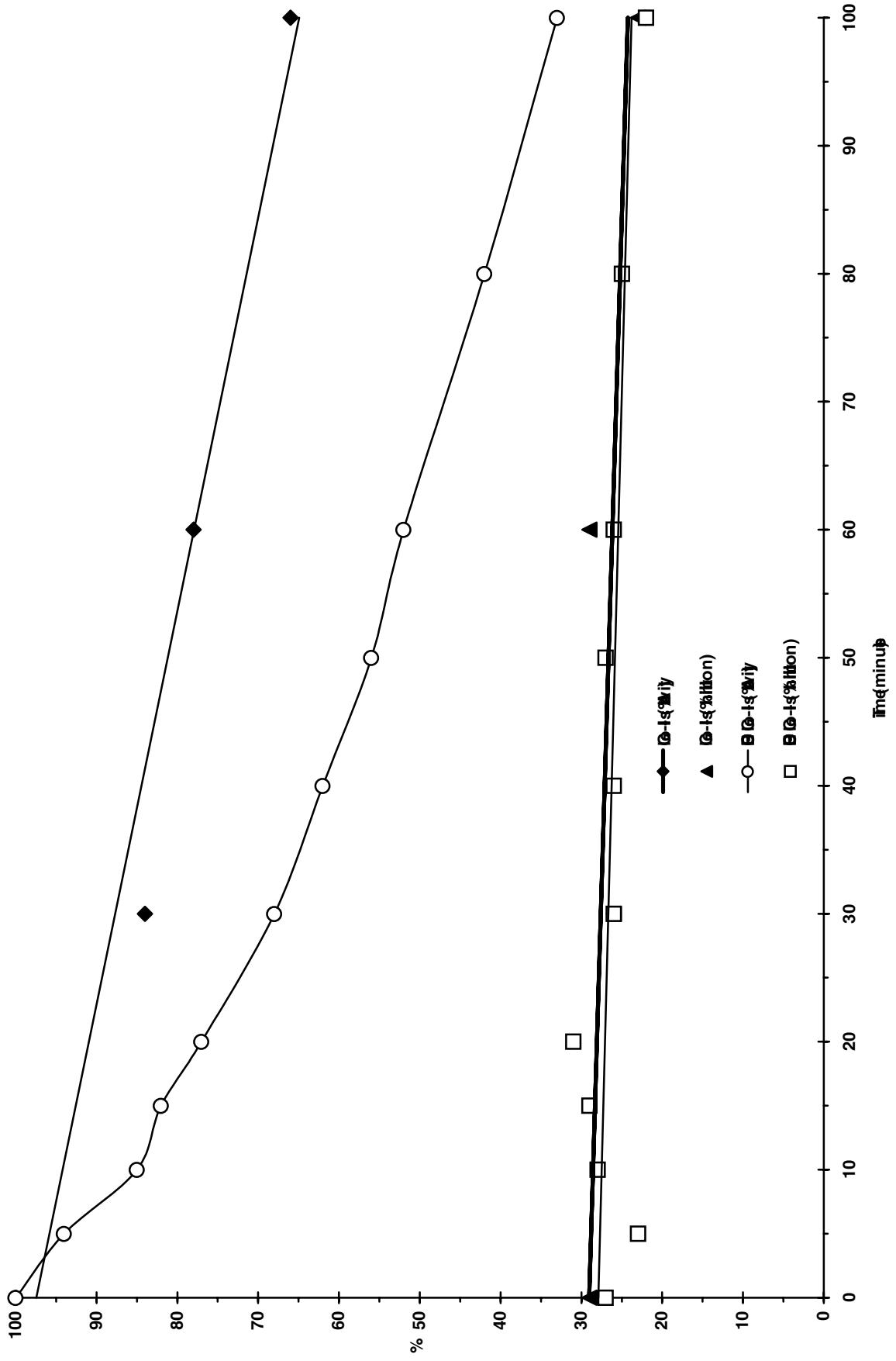

100
90
80
70
60
% 50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100

Figure 29; Effect of Arginine Modification by Phenylglyoxal on Glo-II-s

Chemical modification of arginine residues was done at 1°C using 1-4 IU of Glo-II-s in 100 mM TRIS, pH 7.7. The modification reaction was started by the addition an aliquot of phenylglyoxal / DMSO stock solution (5% DMSO, 5 mM phenylglyoxal final concentration). Aliquots were removed and assays were conducted as previously described, without and without 2 mM ATP added. A control was treated similarly (addition of DMSO without phenylglyoxal). No change was observed in the control solution (data not shown for clarity). Results were recorded as the residual % activity and the residual % inhibition as compared to that of a control sample of enzyme (no modifier added). These values were plotted against the time of incubation. Lines were generated using a computerized linear regression program.

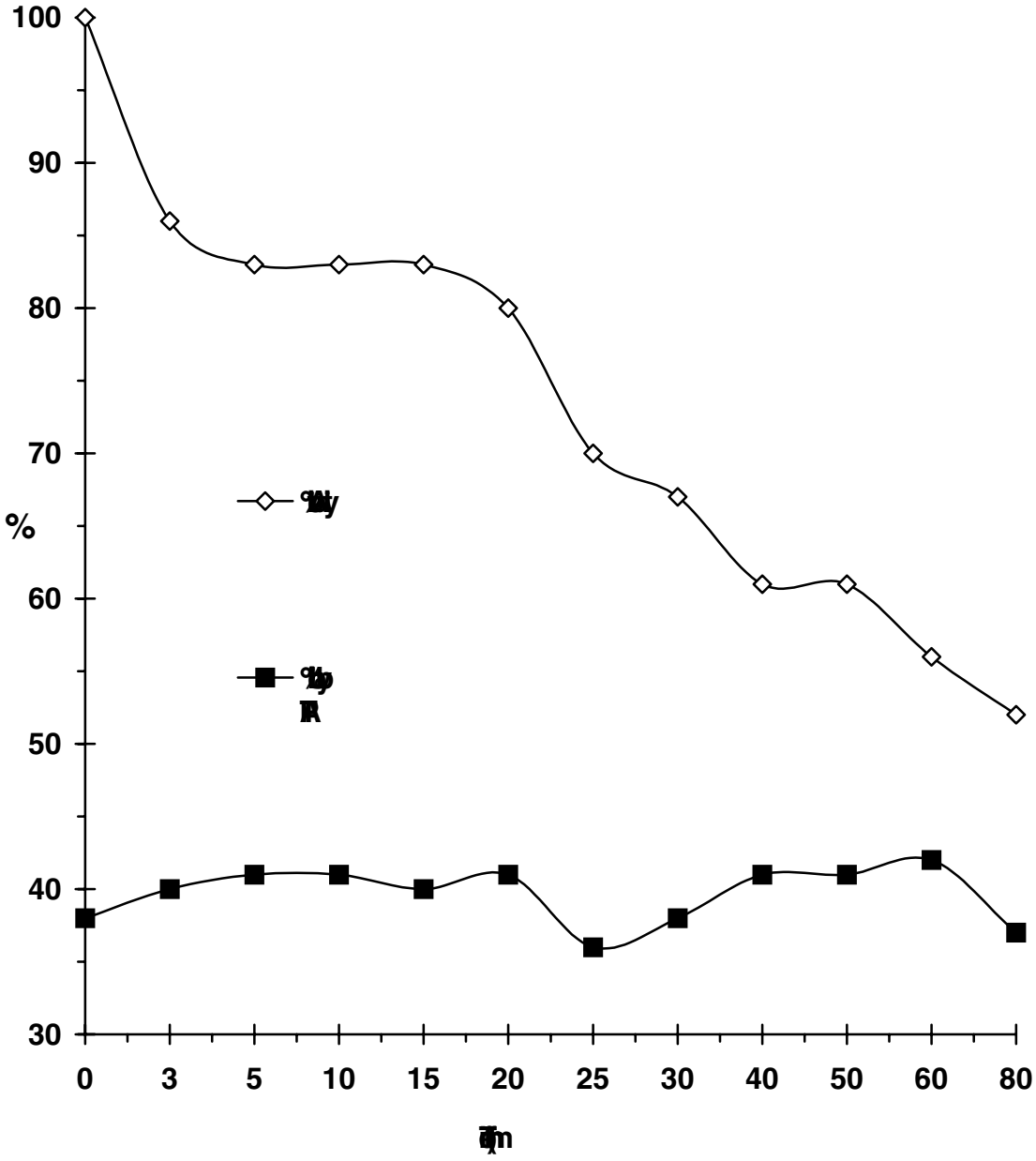

100
90
80
70
60
50
40
30
%
0
3
5
10
15
20
25
30
40
50
60
80

Figure 30; Effect of Sulfhydryl Group Modification on Glo-II-s

Concentrated solutions of Glo-II-s (50 U / ml) containing 5 mM concentrations of either DTNB, NEM, GSH, GSSG, DTA, or diamide were prepared and assayed immediately for both activity and inhibition by ATP. A control sample (no reagent added) was treated similarly. The samples were then incubated for 200 min. at 1°C. Samples were then diluted (1-1250), and assayed in the presence and absence of 2 mM ATP. Results are compared to those of the control. The control underwent no change detectable changes in activity or inhibition by ATP during the incubation. At the end of the incubation period, all samples were diluted similarly and assayed in the same manner. Results are recorded as the % activity or % inhibition remaining after the incubation. The final concentration of the sulfhydryl group reagent in the assay after dilution was 4 μM. Reactions were started with the addition of substrate.

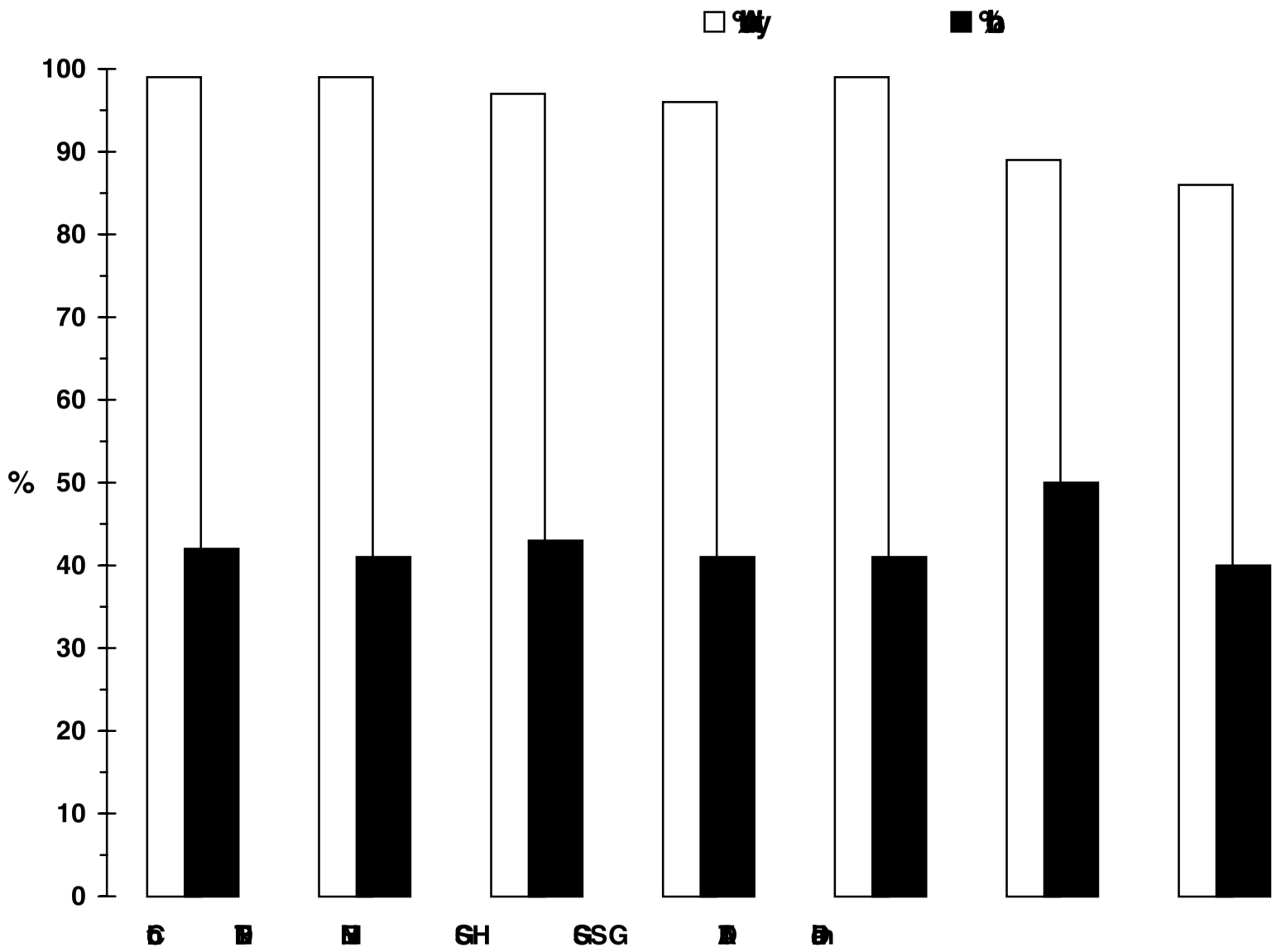

100
90
80
70
60
50
40
30
20
10
0
%

Figure 31; The Effect of DTA on the Activity of Glo-II-i

Incubation of Glo-II-i was done in the presence of 4 mM DTA at 1^0C over 10 hrs. in buffer A. At designated times, aliquots were removed from the incubation and assayed as previously described in the Methods section. Results are compared to a control treated in the same manner.

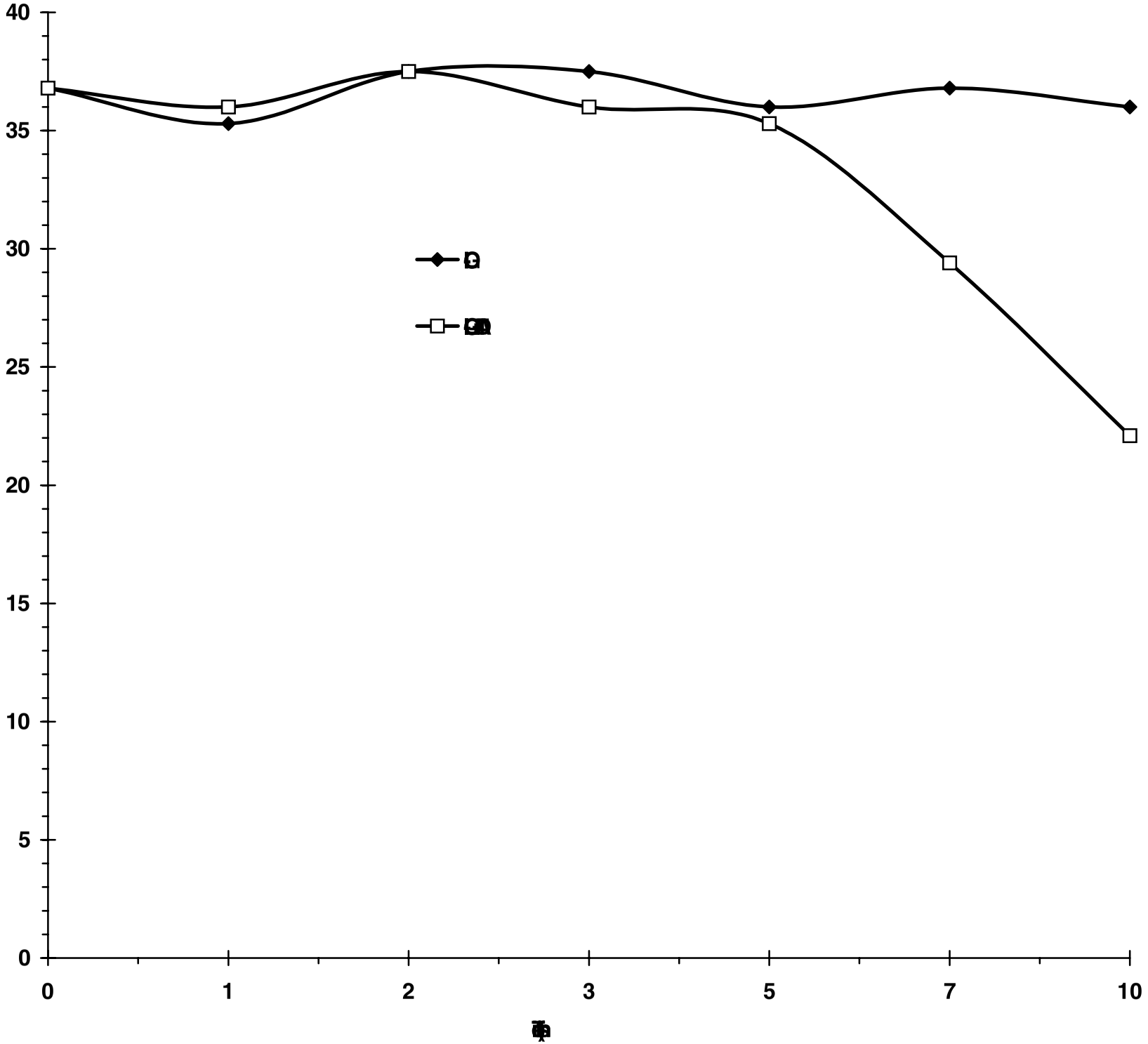

40
35
30
25
20
15
10
5
0
0
1
2
3
5
7
10

Figure 32; The Effect of DTA on the Inhibition of Glo-II-i by ATP

Incubation of Glo-II-i was done in the presence of 4 mM DTA at 1^0C over 10 hrs. in buffer A. At designated times, aliquots were removed from the incubation and assayed as previously described in the Methods section without and with 2 mm ATP. Results are compared to a control treated in the same manner.

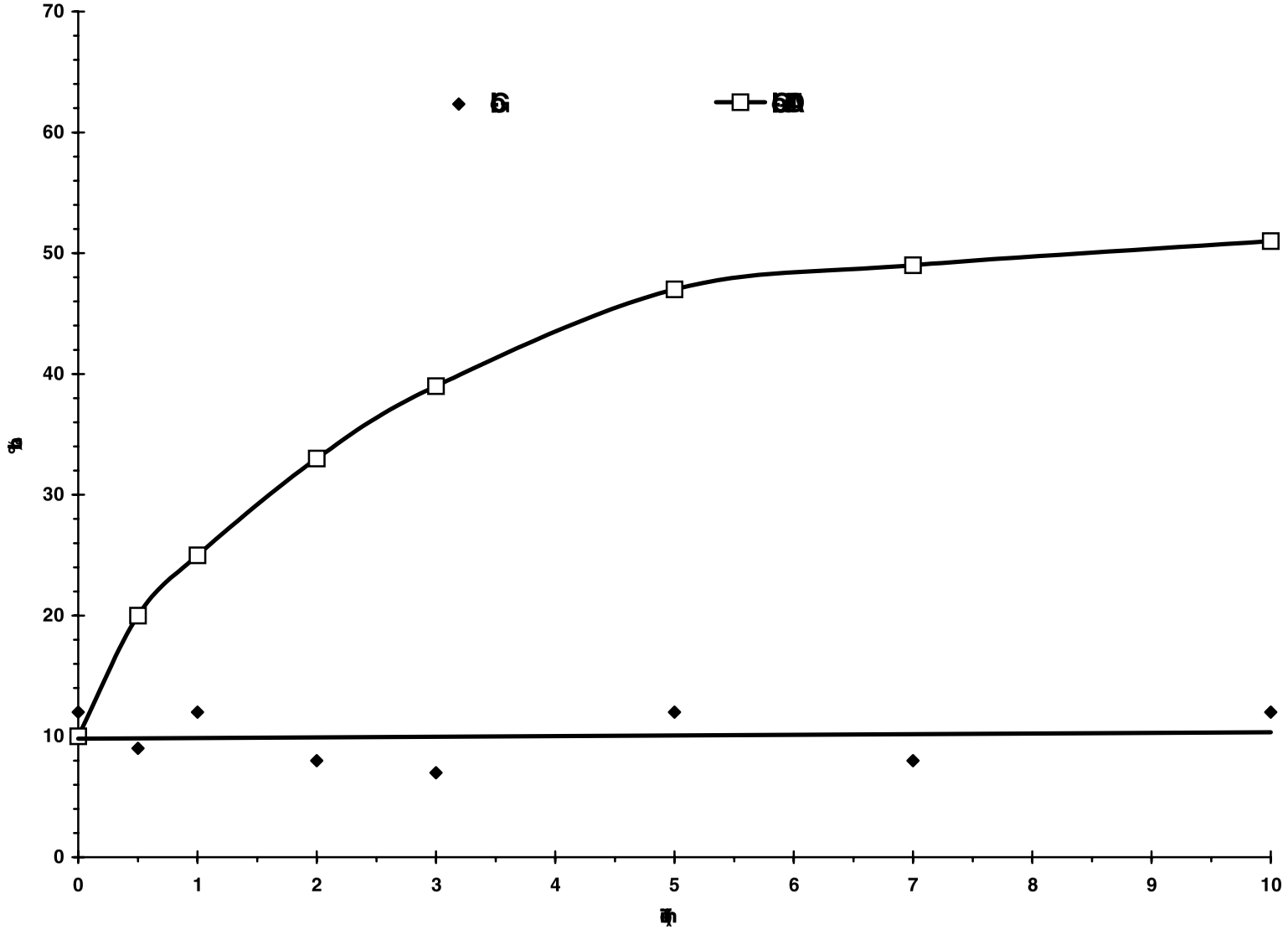

70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10

Figure 33; The Effect of DTA on the Activity of Glo-II-s

Incubation of Glo-II-s was done in the presence of 4 mM DTA at 1^0C over 10 hrs. in buffer A. At designated times, aliquots were removed from the incubation and assayed as previously described in the Methods section. Results are compared to a control treated in the same manner.

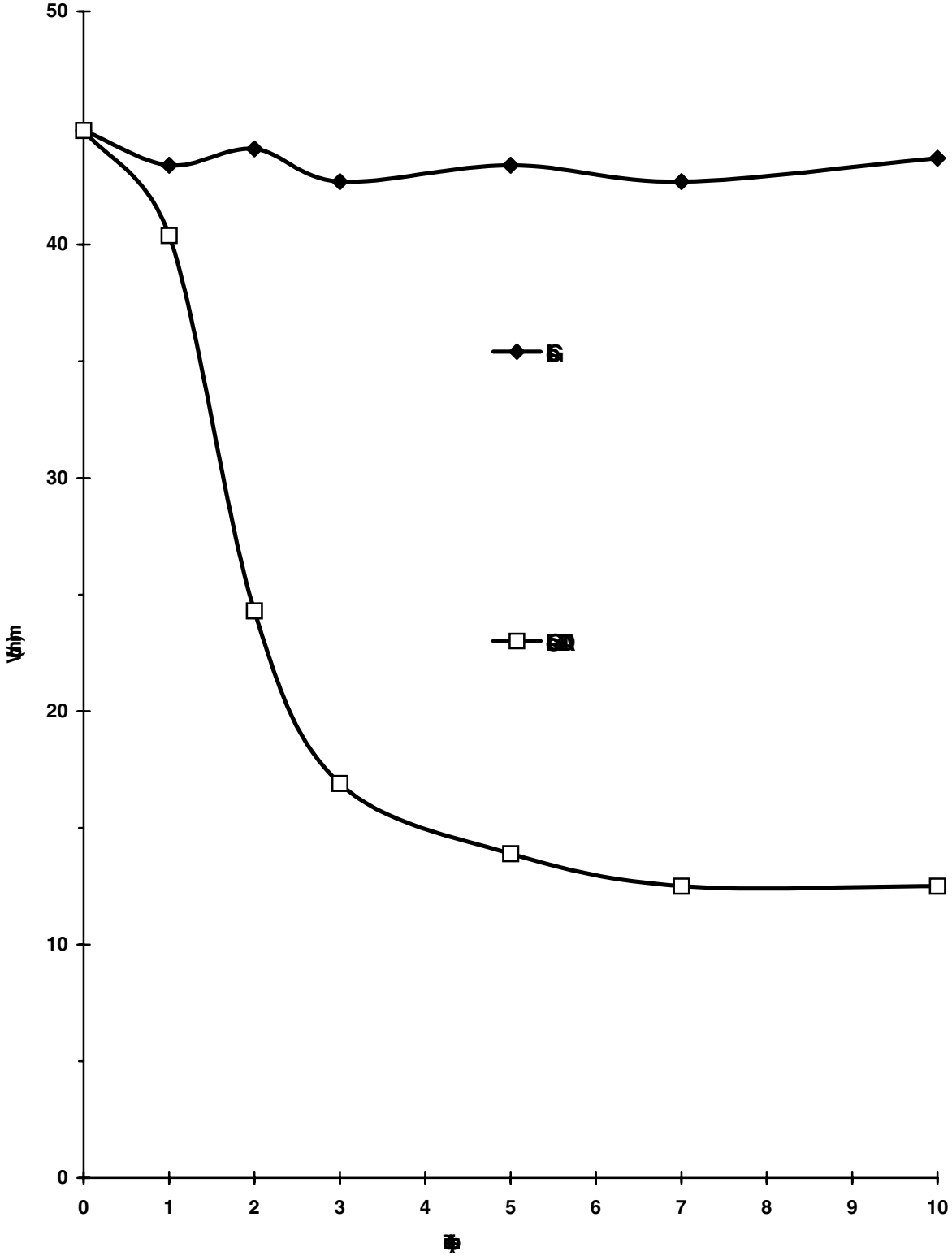

50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10

Figure 34; The Effect of DTA on the Inhibition of Glo-II-s by ATP

Incubation of Glo-II-s was done in the presence of 4 mM DTA at 1^0C over 10 hrs. in buffer A. At designated times, aliquots were removed from the incubation and assayed as previously described in the Methods section without and with 2 mm ATP. Results are compared to a control treated in the same manner.

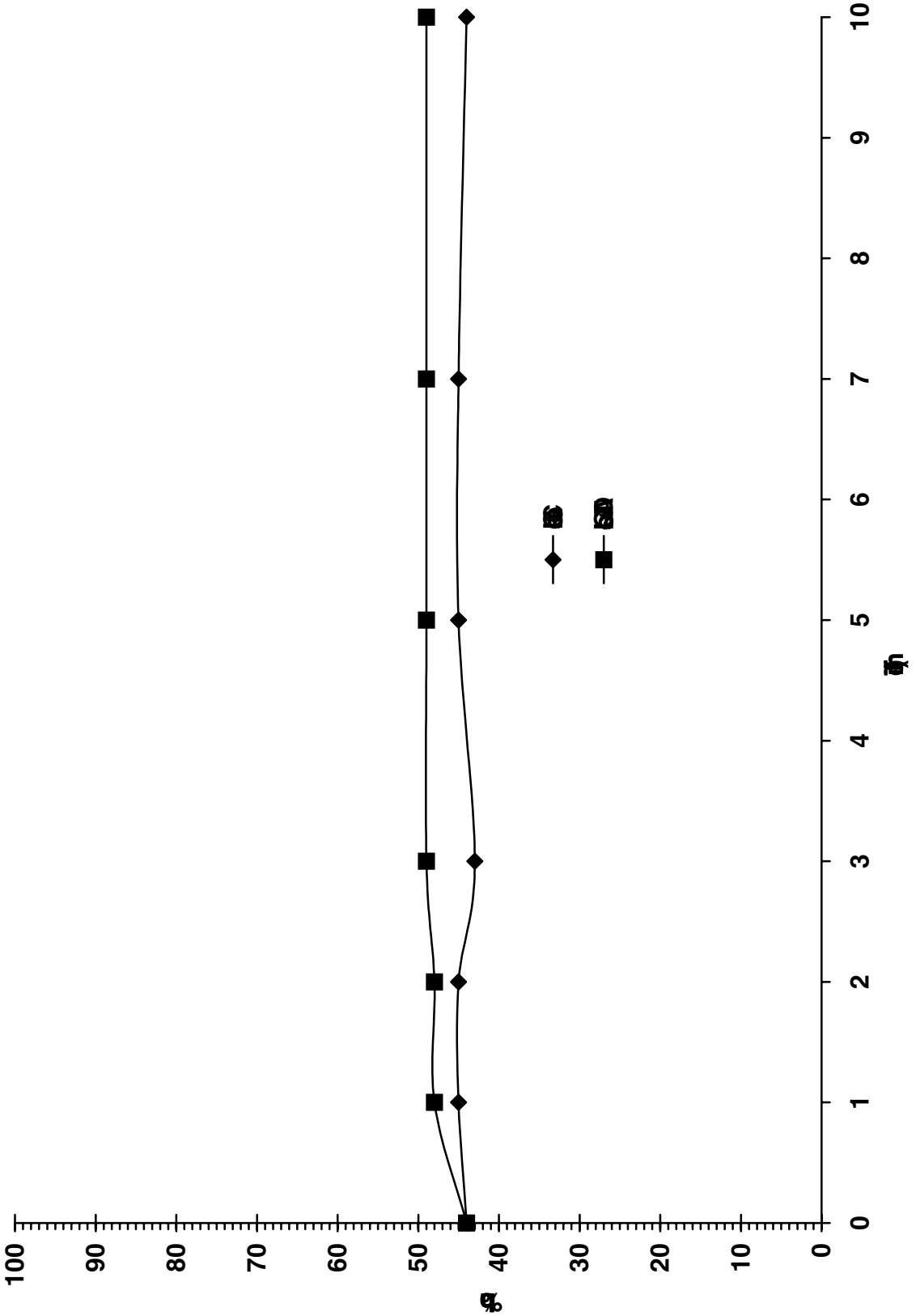

100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10

Figure 35; Lineweaver-Burk Plot of Glo-II Activity after incubation with DTA

The assay method is as described in the Methods section. Velocity is in terms of μmol / min. of GSH produced from SLG. SLG concentrations were from 0.1 to 0.6 mM. ATP concentrations were 0, 0.1, 0.2, 0.6, and 0.8 mM. Lines were drawn using a computerized linear regression program. The Glo-II sample was the Glo-II-s sample after incubation with DTA as previously described.

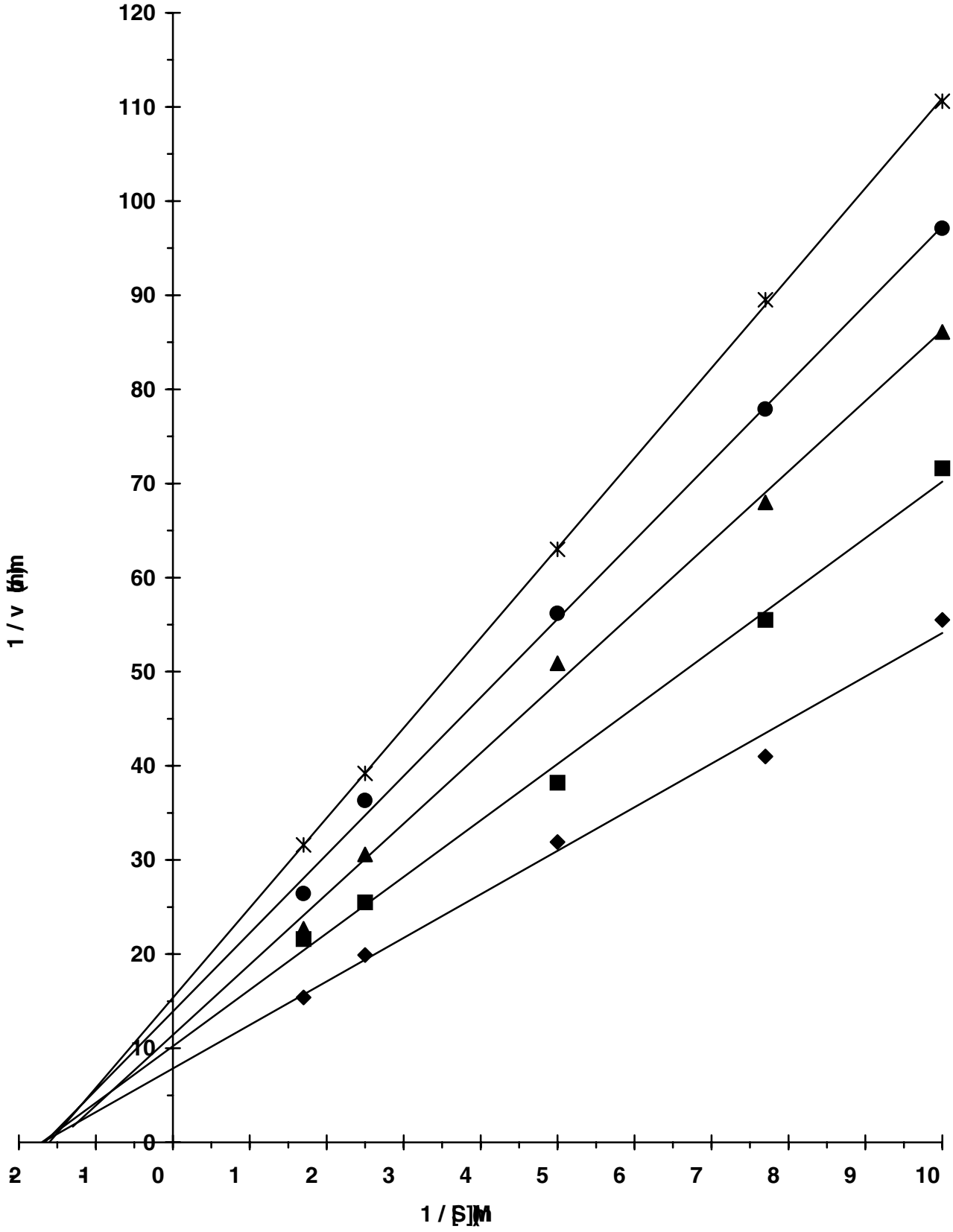

120
110
100
90
80
70
60
50
40
30
20
10
0
-2
-1
0
1
2
3
4
5
6
7
8
9
10

Figure 36; Dixon Plot of Glo-II Activity after Incubation with DTA

The data from Figure 35 was used to construct the plot. Lines were generated using a computerized polynomial regression program.

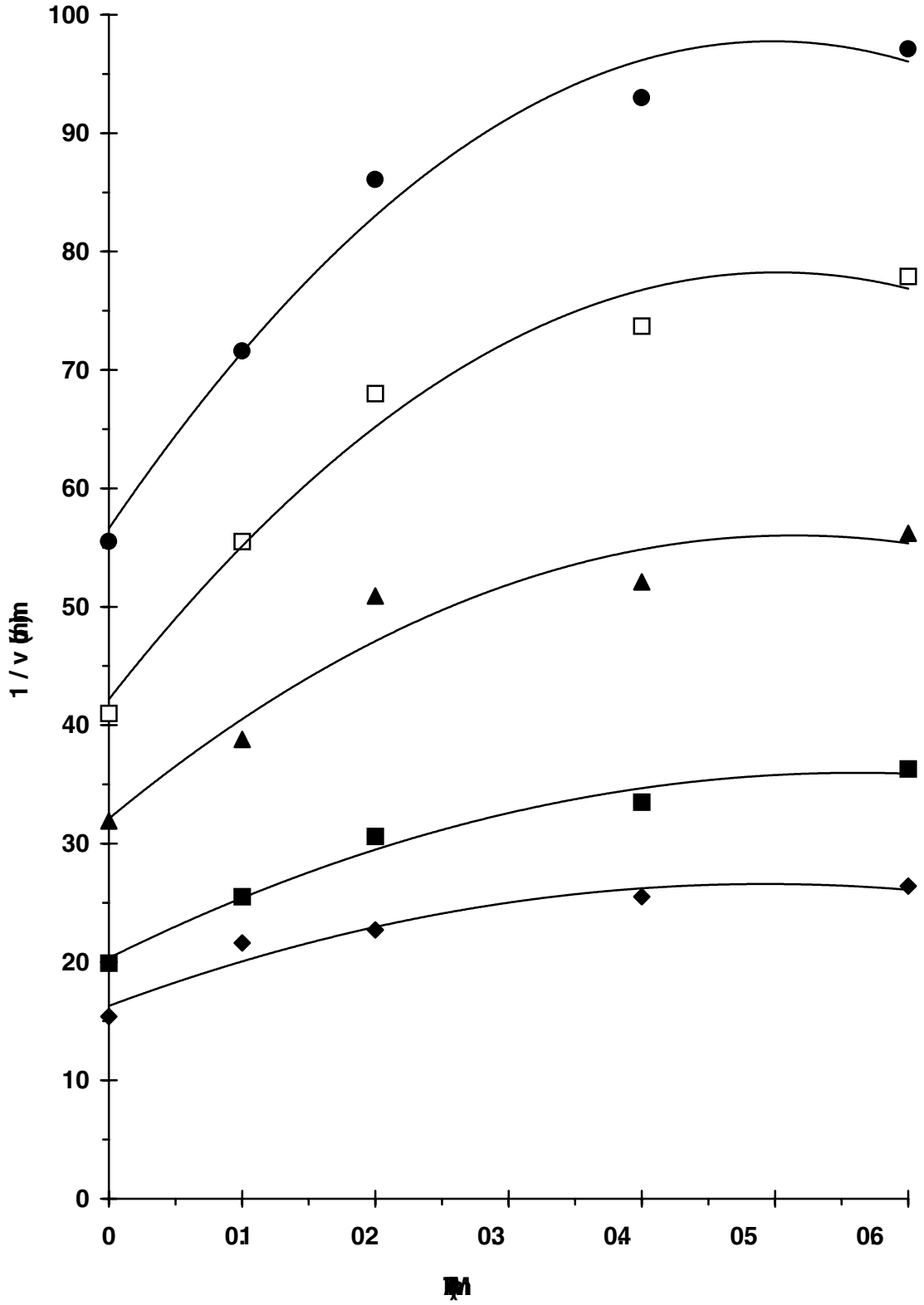

100
90
80
70
60
50
40
30
20
10
0
0
01
02
03
04
05
06

Figure 37; Slope versus Inhibitor Concentration Plot of Glo-II Activity after Incubation with DTA

The data from Figure 35 were used to construct the plot. Lines were generated using a computerized polynomial regression program.

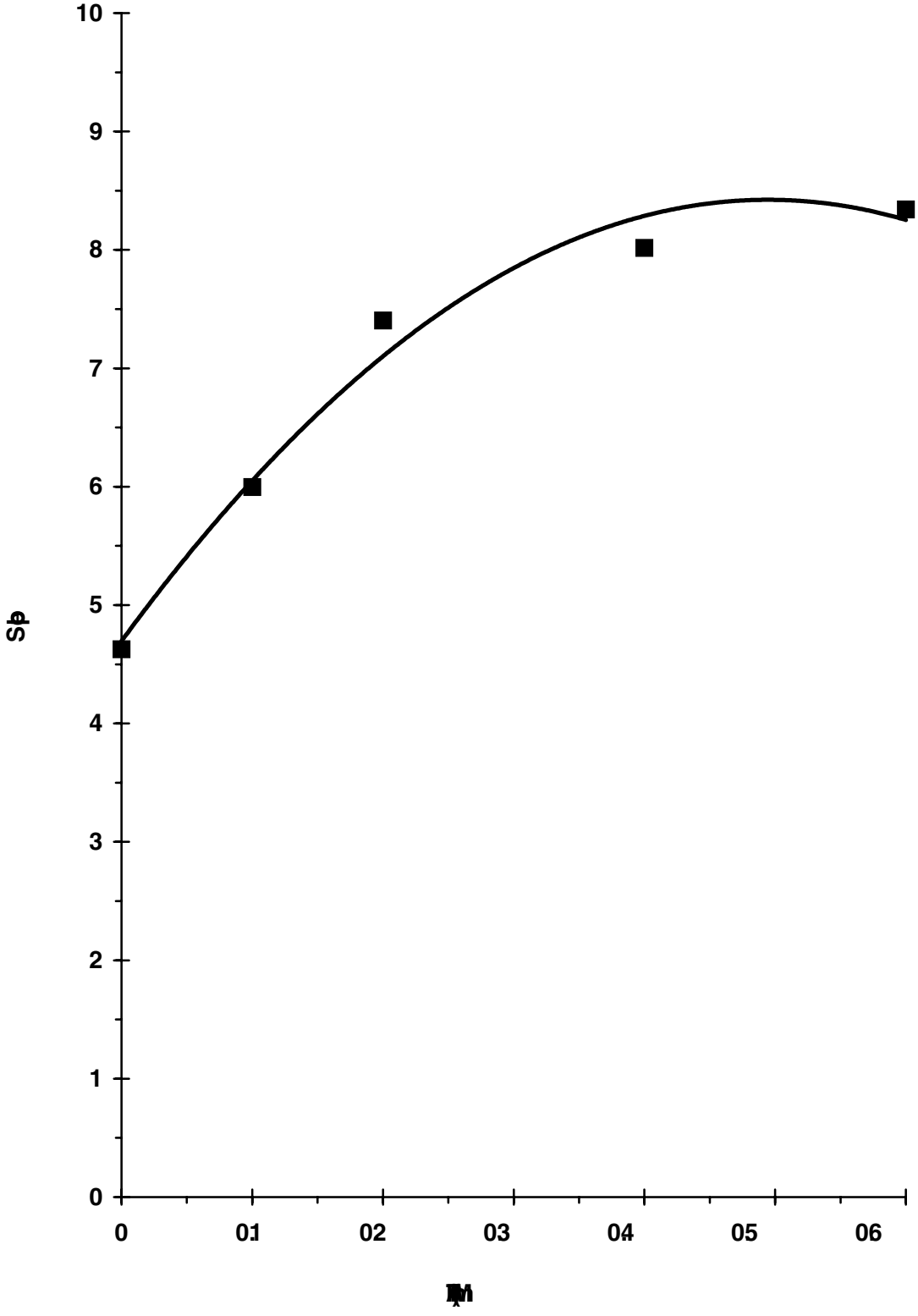
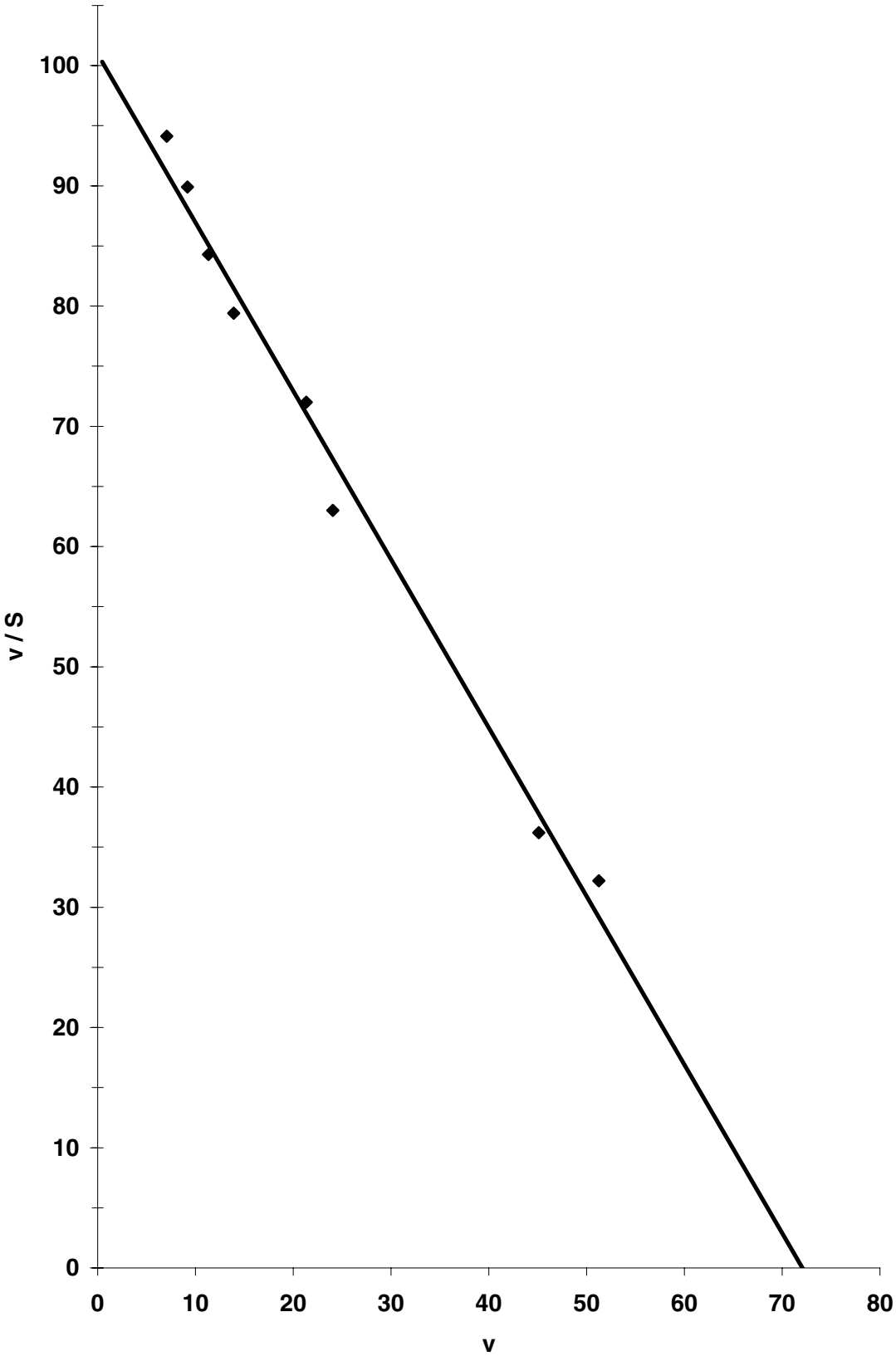

10
9
8
7
6
5
4
3
2
1
0
0
01
02
03
04
05
06

Figure 38; Eadie – Schatchard Plot of Glo-II Activity after Incubation with DTA

Assays were conducted as described in the Methods section. Substrate concentrations used ranged from 0.025 mM to 0.80 mM. Lines were generated using a computerized polynomial regression program. Enzyme velocity (v) is in μmol / min.


100
90
80
70
60
50
40
30
20
10
0
v / S
0
10
20
30
40
50
60
70
80
v

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5. Dakin, H.D., and Dudley, H.W., (1913) J. Biol. Chem. 15, 463.

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